FINAL PROJECT REPORT

PROJECT TITLE:	Characterization of Laccase-like Multicopper Oxidases (LMCOs) in *Arabidopsis thaliana*
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REPORTING PERIOD:	09/15/03 - 09/14/07
INSTITUTION:	University of Georgia Research Foundation, Inc. 617 Boyd Graduate Studies Research Center University of Georgia Athens, GA 30602-7411
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Summary:

Laccase-like multicopper oxidases (LMCOs) have repeatedly been associated with the process of lignification in plants, and work performed in this laboratory prior to initiation of this project suggested that these enzymes might be acting as specific marker for highly localized, small-scale lignification events in tissues not typically thought of as lignified. However, plant LMCOs typically occur as members of gene families and different family members can display disparate enzyme activities that must be taken into account when proposing physiological functions. Further complicating matters, different LMCO gene family members had been shown to have overlapping patterns of expression in bulk tissues. This could indicate a certain amount of functional degeneracy for these isoenzymes, but there was little of no data available on the expression of multiple LMCO genes at the cellular level.

The project proposal called for assessing a selection of *Arabidopsis thaliana* LMCO family members with respect to their enzyme activity, their patterns of tissue- and cell-specific expression, and their subcellular localization, as well as phenotypic changes in loss-of-function mutants, in an attempt to identify specific physiological functions for each of the gene family members investigated.

Specific objectives proposed for the project were as follows:

1) Use of laccase promoter:reporter gene fusions to demonstrate tissue-specific patterns of laccase gene expression at the cellular level in transgenic Arabidopsis;
2) Study LMCO knockouts for clues to physiological functions for these genes;
3) Heterologous expression of the remaining LMCO cDNAs in yeast (preferable) or a plant cell culture system, followed by testing for phenoloxidase, ferroxidase, copper oxidase, and manganese oxidase activities;
4) Demonstration of specific subcellular localization of laccase isozymes using chimeric laccase:GFP fusion proteins, and if time permits;
5) Attempt to identify proteins that associate with LMCOs, provided time and resources.

Research hypotheses proposed for testing in the project were:

1) *Arabidopsis* LMCOs are always associated with cells or tissues that become lignified at some developmental stage;
2) One of the root-expressed LMCOs other than At2g30210 will be associated with lignification events in the Casparian strip of Arabidopsis root exodermis; and
3) Specific *Arabidopsis* LMCOs harbor metal oxidase activity, and take part in metal metabolism.

Much of the early work in this project focused on detailed analyses of the At2g30210 LMCO gene and its product. Results from this project indicate that the At2g30210 gene product plays an extracellular role in the formation of suberin in endodermal cell walls containing a Casparian strip, particularly those that initiate formation in the elongation zone to maturation zone transition of primary root tips**.** This work is the first to suggest a relationship between specific LMCOs and aspects suberin formation.

Subsequent studies examined additional *A. thaliana* LMCO genes, including At2g29130, At5g01190, At5g05390, and At5g58910. The products of these genes were all associated with cells containing either suberin or lignin, but loss-of-function mutants in individual genes did not lead to obvious phenotypes. Overlapping expression of these genes suggests degeneracy in their activity, which means that stacking of multiple loss-of-function mutations will likely be required to define their functions.

Significant Findings:

1. ***The At2g30210 LMCO harbors strong phenoloxidase activity, but no detectable ferroxidase or other metal oxidase activity.***

   At the time the original proposal was submitted we had demonstrated expression of the At2g30210 protein to high levels in *E. coli.* However, we were not able to recover active enzyme, nor were we able to reconstitute activity in the heterologously expressed protein using protocols developed for metal-replacement studies of *Rhus vernicifera* laccase, the archetype LMCO. We subsequently prepared appropriate constructs for expression of the gene in plant cells, and isolated several lines of transgenic tobacco (BY2) cells that produced measurable amounts of active phenoloxidase. None of the cell lines produced large quantities of the enzyme or secreted freely soluble enzyme to the medium. After considerable effort, we were able to recover cellular extracts several tens of micrograms of partially purified enzyme that were tested using multiple assays for phenoloxidase, as well as ferroxidase and metal oxidase, activities. Although the enzyme proved highly active as a phenoloxidase (Fig. 1), no ferroxidase, copper oxidase or manganese oxidase activity was detected, even when the enzyme was added to the assays in great excess over the positive controls. Although the remote possibility remains that the At2g30210 LMCO might be able to oxidize metal ion substrates if properly assisted by unknown ancillary proteins, the simplest explanation of our results is that the enzyme is primarily a phenoloxidase and carries out its physiological function(s) in Arabidopsis via this activity.

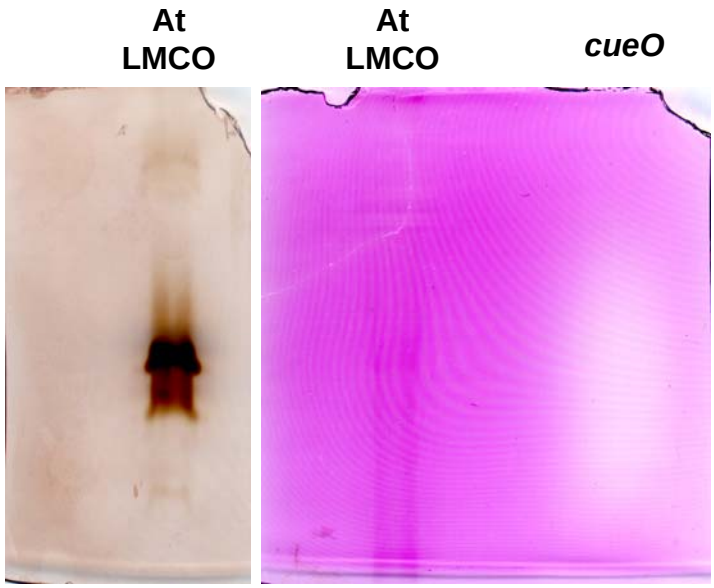

Figure 1. Zymogram analysis of the At2g30210 gene product. Partially purified protein (20 μg) from a single transformed BY2 cell line (AtLMCO) was tested for phenoloxidase activity using 1,8-diaminonaphthalene (left panel). For testing ferroxidase activity (right panel), the amount of LMCO loaded on the gel (200 μg, AtLMCO) was 10x what was loaded for phenoloxidase staining. A sample of the *E. coli* LMCO, CueO, harboring an equivalent level of phenoloxidase activity (Lane *cueO*) provided a positive control for ferroxidase activity. Cleared zones representing ferroxidase activity were visualized by applying the ferrous ion chelator, 3-(2-pyridyl)-5,6-bis (4-phenylsulfonic acid)-1,2,4-triazine (ferrozine).

2. ***T-DNA knockouts of At2g30210 show reduced growth, altered root morphology, and a response to sucrose.***

Among the most useful resources available for the study of *Arabidopsis* genes are the collections of T-DNA insert lines developed and cataloged at the Salk Institute and the gene-trap lines developed at Cold Spring Harbor Laboratory. A line was identified in the Salk collection as having an insert in the last intron of the At2g30210 gene. Upon obtaining a homozygous line for the insert, we found the mutation to be leaky, ostensibly because the T-DNA was spliced out of mature transcripts at a low, but sufficient, frequency for phenotypic escape. However, levels of mature transcript were always less than 30% of levels seen in wild-type Arabidopsis. In the course of working with the Salk T-DNA line, a second insertion mutant was identified among the gene-trap lines produced at CSHL (GT7855). This insertion was in the second exon of the gene, where it resulted in a "strong" allele from which no mature transcripts for At2g30210 could be detected in homozygous lines.

Both knockout lines displayed similar phenotypes, although the CSHL line consistently yielded cleaner, more reproducible results. Neither mutant germinated well in soil (Fig. 2), although seedlings germinated on sterile MS medium and when subsequently transferred to soil grew nearly as well as wild-type. The gene-trap mutant never survived unassisted when it was germinated directly in soil. Because these studies ran in parallel with those described in the preceding section, when we were still testing the LMCO for a possible role in iron metabolism, substantial effort was made to relate growth difficulties in soil to failures in iron metabolism.

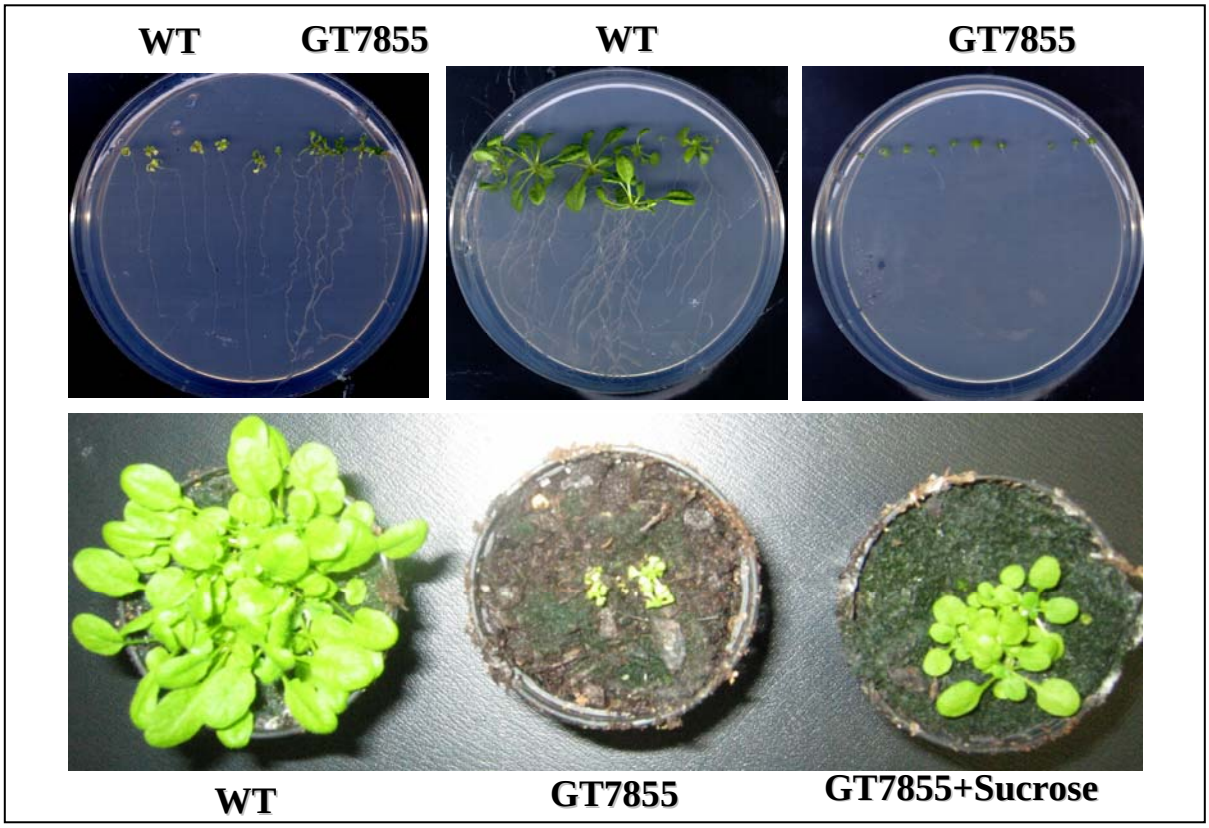

Figure 2. Phenotype of the At2g30210 gene-trap insertion mutant (GT7855). On solid MS medium containing 1% sucrose, the GT7855 mutant showed more lateral root production than wild-type plants (upper left). On MS media without sucrose, growth of the mutant (upper right) was severely repressed compared to wild type (center). In soil (lower panel), poor growth of the mutant in soil (center) could be overcome by watering with dilute sucrose solution (bottom right).

However, a variety of tests with iron supplements and chelators failed to demonstrate significant, reproducible effects on gene expression in wild-type lines or the mutants.

Additional phenotypes were noted when growth of the mutant lines was explored more thoroughly on sterile, tissue culture media (Fig. 2). Germination and root extension in the gene-trap line were normal on MS agar, although the mutant consistently produced a greater number of lateral roots than did wild-type plants under these conditions. Omission of sucrose from the medium, however, led to poor germination and a rapid cessation of radicle elongation in the mutants. Radicle extension and plant growth were resumed if sucrose was added topically to the plants on agar, and similarly, it was determined that watering of germinating mutant seeds with a dilute solution of sucrose could rescue these plants in soil (Fig. 2). Quantitative PCR (qPCR) experiments found that expression of the wild-type gene was repressed by sucrose and induced by salt. Changes in At2g30210 gene expression in response to sucrose were also noted in a paper by Loreti et al. (2005).

3. ***Promoter-reporter constructs enabled demonstration that the At2g30210 gene is most strongly expressed in endodermal cells in the elongation zone of root tips.***

A binary vector in which the At2g30210 promoter was used to drive ß-glucuronidase (GUS) expression was used to transform wild-type Arabidopsis. Transgenic lines expressing this construct clearly showed that the promoter was most active in endodermal cells of developing Arabidopsis roots (Fig. 3). This result matched well with measurements made by Benfey's group using Affymetrix chips, which showed that expression of this gene is strongly up-regulated in those root developmental zones where the endodermis is initially formed (Birnbaum et al. 2003). Whole plant staining also detected localized sites of low-level expression in developing anthers.

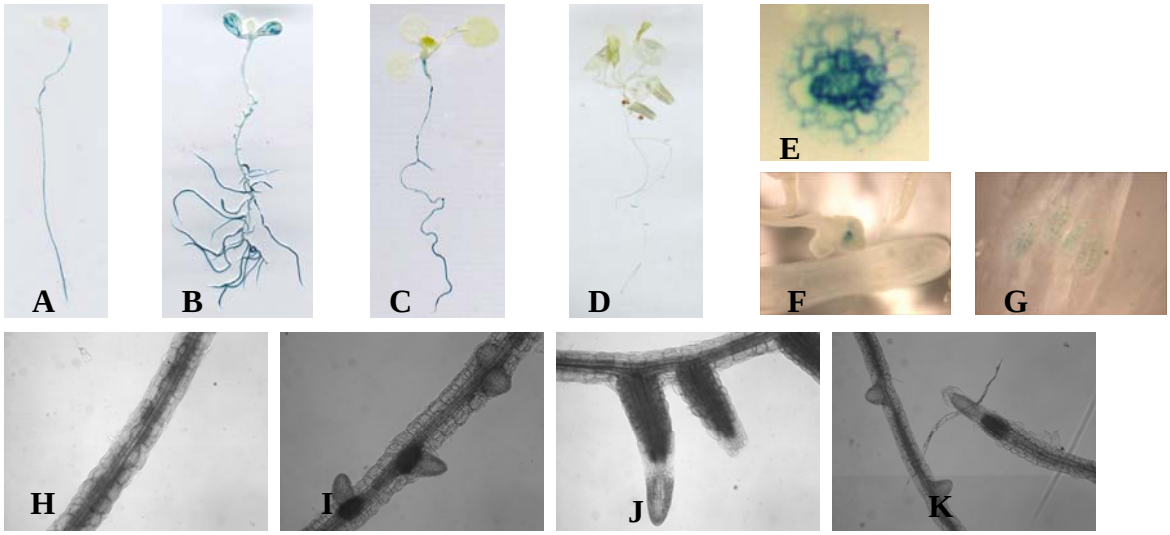

Figure 3. At2g30210 promoter expression patterns in Arabidopsis tissues at different developmental stages. Transgenic seedlings grown in liquid MS medium to ages 1 week (A) and 3 weeks (B), and seedlings grown on MS plates (0.8% agar) to ages 3 weeks (C) and 4 weeks (D), were stained for GUS activity. Endodermal staining is shown in a transverse section from the root tip maturation zone (E). Weak GUS activity is detected in very early stage flowers stained for 3 days. Dissection of the flower from (F) shows faint staining in the pollen sacs (G). GUS activity (dark stained regions, H-K) is shown in a photo series showing lateral roots at various developmental stages in a selected seedling.

4. ***Transgenic Arabidopsis plants expressing chimeric At2g30210 LMCO:mGFP showed that the protein is localized to the cell wall.***

To examine subcellular localization of the At2g30210 gene product, we prepared a chimeric protein in which a modified spectrum derivative of green fluorescent protein (mGFP, specifically YFP; Zhong et al. 2005) was linked in-frame to the carboxyl-terminal end of the At2g30210 gene product. The resultant construct, driven with a 35S promoter was transiently expressed in suspension-cultured tobacco cells (BY2), where the protein appeared to be localized to the cell wall. The same construct was used to transform *Arabidopsis*, and confocal laser microscopy of intact roots from the resulting transgenic plants appeared to support wall localization of the enzyme *in planta* (Fig. 4). Deformation in this pattern was not detected when the tissues were subjected to hypertonic conditions suggesting that the chimeric protein was localized to the cell wall and not the plasmalemma. To determine whether the protein is precisely targeted to the Casparian strip in endodermal cells, we prepared a modified vector in which the 35S promoter was replaced with the At2g30210 promoter. However, we were unable to detect a signal in any of several transgenic lines generated using that particular construct.

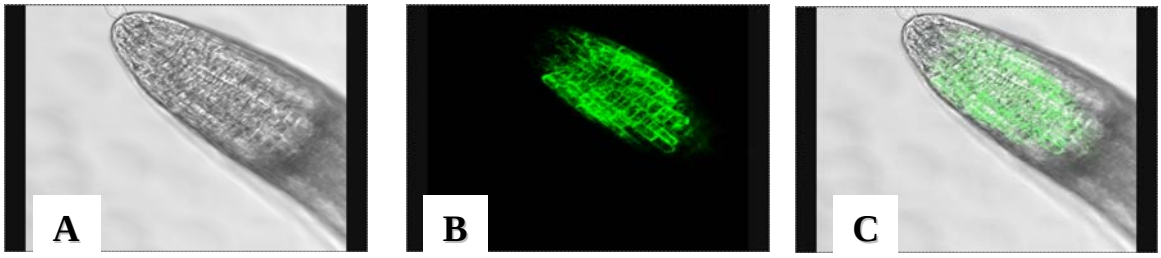

Figure 4. Subcellular localization of the At2g30210-YFP chimeric protein in transgenic *Arabidopsis*. Root tips from 5-day old seedlings were observed using confocal microscopy. (A) Transmission view of a transgenic root tip. (B) YFP fluorescence, shown in green, was localized to the cell periphery, most likely the cell wall, where the LMCO could be involved formation of lignin. (C) Overlap of images A and B.

5. ***The At5g58910 LMCO is expressed to moderate levels in Arabidopsis radicles.***

At the start of this project, the At5g58910 LMCO gene was selected for study in parallel with the At2g30210 gene because both genes encoded unique amino acid motifs in the signal sequences of their products that we hypothesized might confer similar patterns of intracellular localization. Once localization experiments with At2g30210 demonstrated extracellular localization of the gene product, our working hypothesis for localization was disproved, and further work on At5g58910 was given a lower priority. However, lines from Salk and CSHL in which T-DNA and genetrap inserts that should act as knockouts of At5g58910 were identified, and we obtained homozygous lines for the two insertion lines. No obvious phenotypes were associated with these knockouts. Previous qPCR experiments in our lab failed to detect At5g58910 expression in any tissues of *Arabidopsis* (McCaig at el. 2005); however, Birnbaum et al. (2003) and metadata analyses of Affymetrix GeneChip data (https://www.genevestigator.ethz.ch/gv/index.jsp; Zimmermann et al. 2004) indicated low levels of expression for this genes in young roots and mid-stage seeds, respectively. Transcriptional profiling using MPSS (Meyers et al. 2004) suggested that At5g58910 might be expressed in juvenile roots. We were able to demonstrate using GUS reporter constructs in transgenic Arabidopsis that the At5g58910 promoter drove gene expression in the radicle of germinating seedlings (Fig. 5). High-resolution microscopy has so far proven inconclusive in demonstrating whether expression of this promoter is limited to a subset of cells within the radicle.

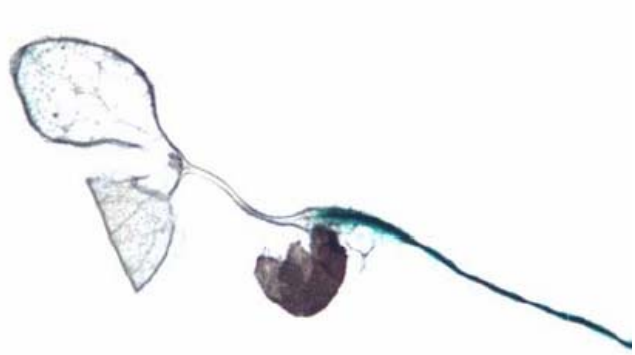

Figure 5. GUS staining shows expression patterns conferred by the At5g58910 promoter.

6. ***Overlapping expression patterns for the At2g29130, At5g01190, At5g05390 LMCO genes.***
A Ph.D. student who joined the lab during Years 3 and 4 of the project, initiated studies of a trio of LMCO genes that the previously mentioned qPCR (McCaig et al. 2004) and GeneChip meta-analyses suggested had overlapping expression patterns in the lignifying tissues of inflorescence stalks. Our previous work had also determined that these three genes fell into three different phylogenetic clades. RT-PCR analyses indicated that all three genes were induced to some degree in excised inflorescence stalks incubated with a 1% sucrose solution (Fig. 6), but not in response to wounding (*data not shown*). Interestingly, induction of LMCO gene expression by sucrose in this case is opposite of the situation found for the At2g30210 root tip gene, whose expression was repressed.

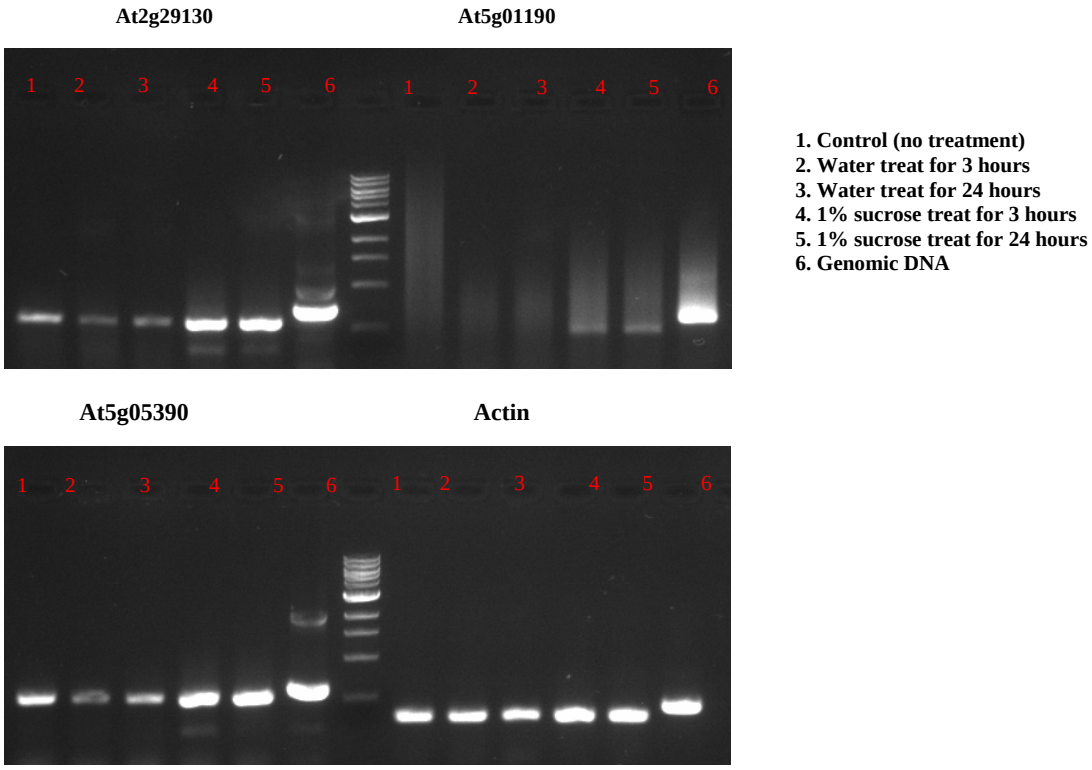

Figure 6. RT-PCR analysis of LMCO gene expression in Arabidopsis inflorescence stalks incubated in water or sucrose solution for varying periods of time. All three LMCO genes showed increased expression in tissues incubated with sucrose.

To examine the tissue-specificity of the promoters driving expression of the At2g29130, At5g01190, At5g05390 LMCO genes, approximately 1.5 kB of genomic DNA upstream of the translational start site of each gene was substituted for the At2g30210 promoter in the construct described in section #3 (above). The At2g29130 promoter drove GUS expression in vascular cells of seedling roots and leaves (Fig. 7), as well as the xylem tissues in inflorescence stalks. Expression was also found at the base and tip of early stage siliques. A blotchy pattern of expression was always seen in leaves, but the stain was always associated with xylem cells in the vascular entworks and there was no sign that the blotches were induced in response to insects or diseases that might have entered the growth room in which plants were cultured.

Figure 8 shows the expression pattern of the At5g01190 promoter. Blotchy, irregular staining of xylem cells in inflorescence stalks and cauline leaves, similar to what was seen with the At2g29130 promoter, can be seen in Panel A. GUS staining in cross-sections of inflorescence stalks was strongest in xylem cells (Panel B), but not necessarily all the lignified fiber cells that encircle the pith tissues.

In plants expressing the At5g05390 promoter construct (Fig. 9), GUS expression was associated with the same tissues (primarily xylem cells) as was seen for the other two LMCO promoters tested in this group, although there appeared to be subtle differences in intensity and precise patterning between lines carrying the three promoter constructs. Thus, particularly strong staining was seen with the At5g05390 promoter construct in the root:shoot junction of seedlings (Fig. 9A), and the vascular junction at the base of siliques (Fig. 9C). Cauline leaves also displayed intensely stained blotches associated with xylem vascular cells (Fig. 9B).

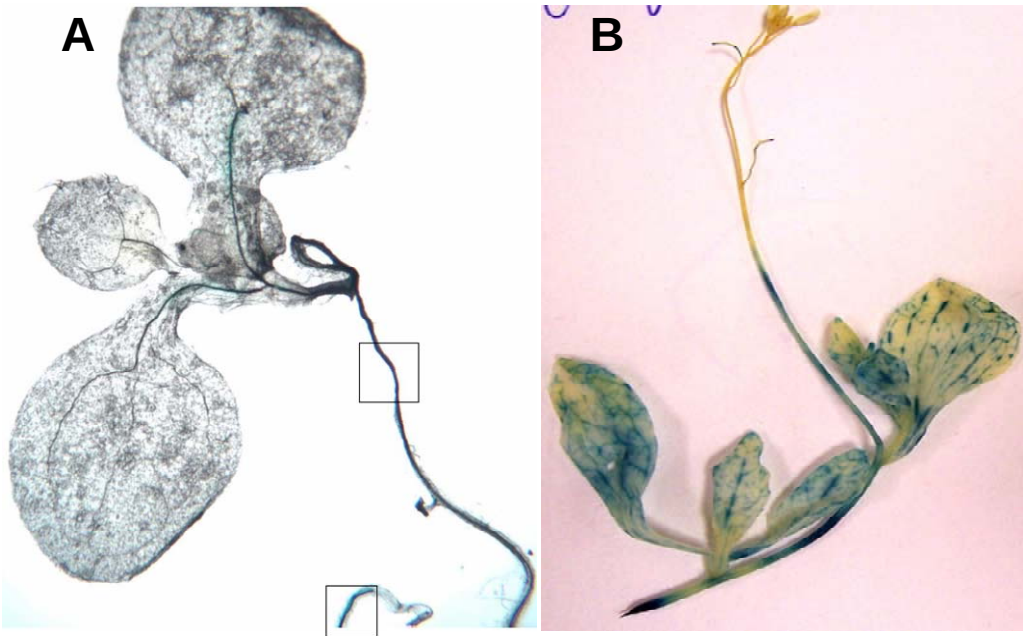

Figure 7. Tissue-specific staining patterns in transgenic Arabidopsis tissues expressing the GUS reporter gene from the At2g29130 promoter. GUS staining was apparent in the xylem cells of roots and leaves in 8-day old seedlings (Panel A). Staining was particularly intense around the root:shoot junction. A blotchy GUS staining pattern was closely associated xylem cells in inflorescence stalks and cauline leaves, as well as the base and tip of developing siliques (Panel B).

Figure 8. Tissue-specific staining patterns in transgenic Arabidopsis tissues expressing the GUS reporter gene from the At5g01190 promoter. All three LMCO genes showed increased expression in tissues incubated with sucrose.

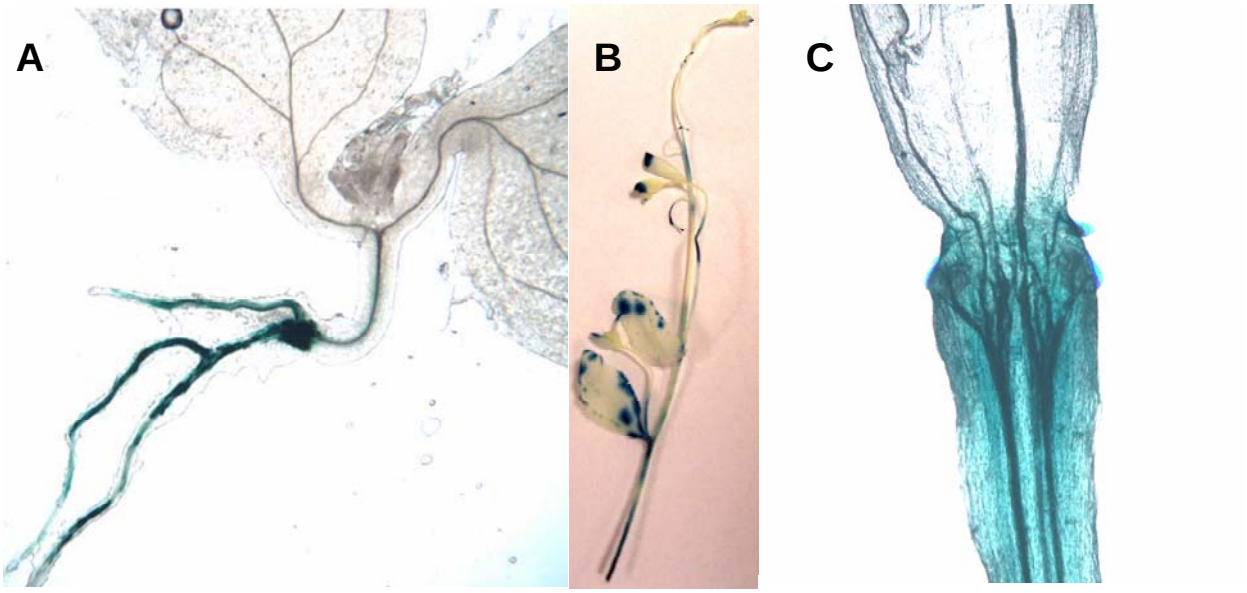

Figure 9. Tissue-specific staining patterns in transgenic Arabidopsis tissues expressing the GUS reporter gene from the At5g05390 promoter. All three LMCO genes showed increased expression in tissues incubated with sucrose.

Homozygous knockout mutants for all three of these LMCO genes (At2g29130, At5g01190, At5g05390) were obtained from the Salk T-DNA insert collection; however, no obvious phenotypes were associated with any of the mutants under normal growth conditions. Given the overlapping expression patterns observed for the gene promoters, it seems highly likely that there is capacity for compensation in the single-gene mutants, and it may require stacking of double- or triple-knockouts to create obvious phenotypes.

Unfortunately, the Ph.D. student that initiated the work on these three inflorescence stalk-associated LMCO promoters suddenly left the graduate training program for personal reasons (her husband was transferred to a new job in Chicago). Consequently, no work to develop stacked, multiple LMCO mutants has been performed, and publication-quality photomicrographs of the GUS expression patterns have not yet been obtained for certain tissues. However, seed stocks for single-mutant knockouts have been placed in appropriate storage and it is hoped that a new student can be recruited to satisfactorily complete these studies.

Conclusions

Results from our studies of the At2g30210 gene are described in a pair of papers that have been submitted for peer-review. Although the results disproved our iron-uptake hypothesis for the physiological function of this gene product, we are quite excited by the possible linkage between this particular gene and suberin biosynthesis. Cells of the root endodermis are characterized by anticlinal walls that are tightly bound together by a narrow band of suberin (Zeier et al. 1999), which constitutes a structure known a Casparian strip. The Casparian strip functions to seal the perimeter of the endodermal walls and, thereby, create an apoplastic transport barrier to water, ions, and sugar separating the root cortex and stele (Sattelmacher 2001). This brings these solutes and water under cellular control, as they must pass through membranes (symplasm) to move between the cortex and stele. Changes in structure or composition of the Casparian strip are known to impact hydraulic conductivity and ion flow (Schreiber et al. 2005) in ways that would be consistent with the phenotypes we have recorded in the At2g30210 mutants.

Although we've previously documented a tight link between LMCO activity and lignin deposition, this is the first observation of a possible link between these enzymes and suberin formation. However, such a link is reasonable from what we know of suberin composition. That suberin actually contains a polyphenolic core resembling lignin has been accepted since the seminal work of Kolattukudy (1981), although recent compositional analyses suggest that non-traditional phenolic units are frequently incorporated into the polymer, and thus the polymer may or may not be classified as "true" lignin (Bernards 2002). Although peroxidases have frequently been implicated in the polymerization events related to suberization (Quiroga et al. 2000, Bernards and Razem 2001), no one has previously associated LMCOs with suberizing cell walls. In addition to the endodermis, other root tissues containing suberin include the epidermis, the exodermis, and the root phellem, as well as bundle sheath cells, cork (phellem) and other aerial portions of the plant (Bernards 2002). Our results from the study of the At2g30210 gene suggest that all of these cells and tissues may be sites where we could expect LMCOs to be expressed. Casparian strips are well-documented features of the root endodermis and exodermis, but are less well-known for their presence in a variety of specialized leaf, stem, and glandular structures, such as nectaries and salt glands (Lersten 1997). In these specialized cases, they are typically restricted to relatively rare, highly specialized cells that use energy-dependent systems to pump metabolites against concentration gradients. As a consequence of all this, we are excited by the possibility that some of the other LMCOs expressed in *Arabidopsis* will be associated with Casparian strip formation. In fact, the expression patterns detected for several of the LMCO promoters in which strong GUS staining was associated with transition tissues, such as the root:shoot junction, as well as the base of flowers and siliques, would be consistent with known Casparian strip-type structures in these tissues.

With regard to the three LMCOs studied for their expression in inflorescence stalks (At2g29130, At5g01190, At5g05390), their close association with lignified tracheary elements in xylem tissues of the stalks, as well as the vascular networks in cauline leaves, was consistent with our previous studies in *Zinnia elegans* (Liu et al. 1994). Given the importance of lignification as a contributor to plant stature and

architecture, and the critical roles these features play in reproduction and life history of plants, it is, perhaps, not surprising that there is a high degree of degeneracy built into the system. However, it yet remains to be determined whether specific LMCOs may have preferred or even specific substrates for whose polymerization they are the primary contributor. It may yet remain for high precision metabolomic studies to determine the existence of such precursors.

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Personnel and Training

Much of work on the At2g30210 gene was performed by a graduate student, C-T. Wang, who defended his Ph.D. dissertation in July 2005. Dr. Wang remained on the project as a postdoctoral researcher for a few months beyond the completion of his degree before taking a postdoctoral position with Dr. Amy Brunner at Virginia Tech working in the area of *Populus* genomics. As noted below, two research papers from Dr. Wang's work have been submitted to Plant Physiology, and results of his work were also presented at the annual Arabidopsis Research Conference in Madison, WI.

In the first year of the project, a small amount of funds enabled completion of work on other plant laccases that was initiated under a previous grant from the Energy Bioscience Program. This funding enabled Dr. J.T. Hoopes to complete a successful defense of his Ph.D. dissertation in January 2004. Dr. Hoopes' dissertation project, which was fully funded through Energy Biosceinces over a couple of projects, has so far resulted in two published refereed research articles that noted DOE support. (As listed below, one of these specifically noted funding from this project. A copy is attached to this report.) One additional research paper that notes specific funding from this project has been submitted for review, and a second is currently being revised in response to reviewer comments.

Also in the first year of the project, Dr. Bonnie McCaig joined the project as a postdoctoral researcher and completed the initial analysis of Arabidopsis LMCO gene family structure, and a survey of LMCO family member expression in various Arabidopsis tissues. This study was published in Planta (see below), and acknowledged funding from this project. A copy of the published manuscript is included in this report.

Dr. Shenghua Yuan worked as a postdoctoral researcher on the project in Years 3 and 4. Her primary focus was to prepare an expression vector in which the At2g30210:YFP fusion protein was placed under the control of the At2g30210 promoter, and then generate transgenic Arabidopsis lines expressing the vector. The goal of the work was to determine whether the At2g30210 gene product would be localized specifically to the Casparian strip, rather than the entire wall of endodermal cells, if it was expressed specifically in these cells. Although transgenic lines were isolated in which transcription of the chimeric gene could be detected by PCR, the YFP marker could not be detected by confocal microscopy in any of the lines. Unfortunately, the absence of data could equally well result from at least a couple of different sources. Most likely, the level of expression from the At2g30210 promoter is too weak to provide sufficient protein (versus the original 35S promoter) for detection by the available instrument. Alternatively, perhaps the protein is, indeed, localized to the Casparian strip, but its signal is quenched there by the proximity of polyphenolic materials. While it is unlikely that any of the work completed by Dr. Yuan will result in publications, the valuable training she received on this project did result in her accepting a research position in the Department of Ecology and Evolutionary Biology at Yale University.

As noted in the preceding project report, a third Ph.D. candidate, Ms. Ruobing Wang, was funded for two years from the project, but had to drop from the training program for personal reasons. Although her research results (reported above) are too fragmented for publication at this time, we anticipate recruiting another student who will complete this work to an extent that it can be published. Funding from this DOE project will be duly noted in any future papers including Ms. Wang's results.

Publications Acknowledging DOE Support for This Project

Research Articles

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Original article

Ferroxidase activity in a laccase-like multicopper oxidase from *Liriodendron tulipifera*

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Abstract

Ferroxidase activity was detected in a laccase-like multicopper oxidase (LMCO) produced in transgenic tobacco cells expressing an LMCO cDNA (Lt*lacc2.2*) cloned from yellow-poplar (*Liriodendron tulipifera*). This marks the first report of ferroxidase activity associated with a plant laccase and suggests that some members of this plant enzyme family may have physiological functions based on activities other than their more widely recognized phenoloxidase activity. Recent work with LMCOs from bacteria, yeast and mammals has shown that metal oxidase activities in these enzymes can be important for their primary physiological functions. With respect to ferroxidase activity in certain plant LMCOs, it is proposed that the high levels of LMCO expression in plant vascular tissues may reflect the need for high-efficiency iron uptake pumps in tissues that undergo lignification during normal development. Such iron uptake pumps would function to minimize levels of free iron so that reactive oxygen species do not reach toxic levels when H_2O_2 is generated for peroxidase-mediated monolignol coupling during lignin deposition.

Keywords: Ceruloplasmin; *fet3*; Iron uptake; Laccase; Lignification; Lignin

1. Introduction

Laccase (EC 1.10.3.2), a copper-dependent phenoloxidase isolated from the sap of the Japanese lacquer tree, *Rhus vernicifera* [4,38], was the first characterized member of the enzyme superfamily commonly referred to as the multicopper oxidases (MCOs) [28]. Multiple catalytic copper centers and oxidative activity are the two hallmarks for members of the MCO superfamily, a group of enzymes that has grown rapidly in recent years to contain, in addition to laccase, such other enzymes as ceruloplasmin, tyrosinase, catechol oxidase, methane monooxygenase, sulochrin oxidase, dihydrogeodin oxidase, phenoxazinone synthase, and bilirubin oxidase [26,28]. Enzymes in the MCO family are diverse in both function and structure, and representatives have been identified in eubacteria, fungi, plants and animals [28]. Although some of these enzymes oxidize specific, identified substrates, and have been named accordingly, most of the better known MCOs are capable of oxidizing multiple potential substrates, and this has created significant confusion in enzyme nomenclature [26]. Strictly speaking, the name laccase should be reserved for those members of the MCO family that are found in plant saps containing the unsaturated alkylcatechols ("laccol," also known as "urushiol") that are their natural substrates [10]. However, the name has frequently been used to identify any MCO that contains four copper atoms in a specific arrangement of Type-1, Type-2 and Type-3 electromagnetic centers [28]. In recognition of the multiplicity of functions likely performed by the numerous MCOs appearing in plant genomes, we have elected to abandon our previous use of "laccase" to cover all monomeric, four-copper MCOs in favor of "laccase-like multicopper oxidases" or "LMCOs." Following the traditions of enzyme nomenclature, more descriptive names may be assigned once specific catalytic reactions and metabolic functions are defined for the various LMCOs.

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid); BSA, bovine serum albumin; DAN, 1,8-diaminonaphthalene; LMCO, laccase-like multicopper oxidase; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species.

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LMCOs have been identified in bacteria, fungi and animals [28], where they function in metal oxidation [6,7,20] and lignin degradation [12], as well as detoxification of antimicrobial agents [27]. Additional LMCOs have been cloned and characterized from several plant species [19,22,23,32], yet their physiological functions in these organisms remain unclear [5]. It has been argued that these enzymes may be involved in lignin deposition by virtue of their ability to polymerize monolignols [34], their association with plant vascular tissues [3,25], and the impact that antisense laccase gene expression has on xylem tissue formation [30]. Yet, definitive evidence demonstrating a role for these enzymes as primary catalysts of lignin polymerization remains elusive.

Saccharomyces cerevisiae uses a pair of LMCOs, encoded by the *fet*3 and *fet*5 genes, as components of high-affinity iron uptake systems that regulate flux of this critical metal through the plasmalemma and vacuolar membrane, respectively [2,36]. Like many organisms, *S. cerevisiae* acquires most of its iron in the ferrous state via a relatively non-specific divalent metal transporter (*fet4*) [9]. Yet, when external conditions, such as elevated levels of copper or zinc, lead to unbalanced uptake of divalent metals, expression of the MCO-dependent Fet3 system is induced. The LMCO in this iron "pump" oxidizes the divalent ferrous ion to the trivalent ferric form, which is immediately passed through the membrane by an associated permease [33]. The specificity of this iron uptake system appears to derive at least in part from the paucity of trivalent metals ions circulating in the environment, and it enables yeast to balance iron intake against that of other divalent metal ions. Due to its higher affinity for iron than the Fet4 system, the Fet3 system is also critical for survival of yeast when extracellular iron concentrations drop to very low levels [7].

Discovery of the LMCO-dependent iron uptake systems in yeast has had a profound impact on our understanding of eukaryotic iron metabolism in general [1]. For example, the ferroxidase activity associated with ceruloplasmin, an abundant MCO found in mammalian blood serum, has been proposed to work in conjunction with circulating transferrin to minimize levels of circulating "free" iron, thereby limiting the generation of reactive oxygen species (ROS) [14]. Subsequent work has identified hephaestin, the MCO component of an iron pump that appears to be critical for moving iron from intestinal enterocytes into the circulatory system [37].

This study marks the first demonstration of a ferroxidase activity associated with a plant LMCO. The enzyme in this case is normally expressed in the lignifying xylem tissue of a deciduous hardwood tree, yellow-poplar (*Liriodendron tulipifera*). On the basis of this enzyme's ferroxidase activity, we discusses how its potential involvement in an iron uptake system could represent an alternative to monolignol polymerization as the physiological function for these enzymes in higher plants.

2. Results

2.1. Heterologous expression of laccase

Previous studies from this lab and others have shown that multiple LMCO genes are expressed simultaneously in lignifying plant tissues [19,23,32]. In one of these, four LMCO cDNAs (Lt*lacc2.1, 2.2, 2.3, 2.4*) from the lignifying xylem tissues of yellow-poplar (*L. tulipifera*) were cloned and expressed in suspension-cultured transgenic tobacco cells [23] to make possible the independent characterization of the individual gene products. Unfortunately, in contrast to the stable transgene expression found in tobacco cells transformed with a constitutively expressed LMCO gene from sycamore maple (*Acer pseudoplatanus*) [8], the levels of enzyme activity in tobacco lines transformed with constructs of the yellow-poplar genes under control of a dual CaMV 35S promoter declined rapidly during extended culture. In cell lines receiving the yellow-poplar constructs, activity dropped to nearly undetectable levels within 6 months of the initial transformation event. Continued presence of the LMCO transgenes in cell lines that lost expression was confirmed by PCR analysis of genomic DNA, but RT-PCR showed that gene transcription was lost over time in culture (data not shown). These results were consistent over three independent transformation experiments and more than 60 cell lines that initially expressed one or the other of the four yellow-poplar LMCOs. Adding to the difficulty this presented for obtaining sufficient protein for enzyme characterization, the growth rate (g fresh weight week^{-1}) of transformed cell lines appeared to be inversely proportional to the level of yellow-poplar LMCO activity initially expressed. Those cell lines that expressed the yellow-poplar LMCOs at the highest levels generally grew at no more than half the rate of cells lines transformed with empty vector controls. This was in contrast to tobacco cell lines transformed with the sycamore maple LMCO gene, which continued to grow at the same rate as untransformed cells. These results suggested that the activities of the constitutively expressed yellow-poplar LMCOs might be impeding normal cellular functions in the transformed cell lines.

For the current study, tobacco cells were again transformed with the dual 35S:Lt*lacc2.2* construct for expression of the yellow-poplar enzyme, and kanamycin-resistant cells lines were selected within 6–8 weeks of the bombardment. Cell lines expressing the LMCO transgene were identified by their ability to oxidize a phenoloxidase substrate (0.1% aqueous ABTS) applied directly to the surface of calli. The cells did not release diffusible LMCO activity into the surrounding medium, as was the case for cells expressing the sycamore maple (Ap*lacc1.1*) gene [8]. The cell line expressing the highest level of phenoloxidase activity was expanded to production-scale (300 g cells week^{-1}) within 12 weeks of the original transformation event.

2.2. Detection of ferroxidase activity

Lysates from harvested and pooled cells were concentrated and partially purified by a combination of ammonium sulfate precipitation and ultrafiltration. Overall yield of LMCO phenoloxidase activity from 900 g of transformed cells was 0.12 units. Ferroxidase activity was detected in the concentrated extract using the ferrozine assay described in Section 4. The specific ferroxidase activity in the concentrated enzyme pool was 18.3 nkatal mg^{-1} protein and the apparent K_m of the enzyme for Fe^{2+} was 40 μM. The apparent K_m of the yeast FET-3 protein for Fe^{2+} is about 5 μM [15], while ceruloplasmin displays non-typical, diphasic kinetics with respect to Fe^{2+} such that two K_m values, 0.6 and 50 μM, were determined for high (210 nM) and low (21 nM) concentrations of the enzyme, respectively [29]. The K_m values for ceruloplasmin ferroxidase activity seem particularly relevant for comparison with the yellow-poplar LMCO given that these two enzymes function within the relatively iron-rich milieu of living organisms, while the yeast enzyme must deal with naturally low levels of iron that exist in the environment. In addition, limited amounts of the partially purified yellow-poplar enzyme prevented testing of kinetic parameters at high enzyme concentrations.

Zymogram analysis showed that the ferroxidase and phenoloxidase activities of the yellow-poplar LMCO co-localized in SDS-PAGE gels (Fig. 1). In this gel system, the Lt*lacc2.2* LMCO migrated significantly faster than the Ap*lacc1.1* LMCO, but not quite as quickly as the *Escherichia coli yacK* gene product. Gels stained for phenoloxidase activity in the presence of 5 mM H_2O_2 showed no detectable increase in band intensity or the appearance of additional bands in any of the lanes, verifying that peroxidases were not responsible for any of the bands detected (data not shown). The multiple bands of ferroxidase activity for the *E. coli* enzyme appeared to be artifacts of the gel system since samples subjected to heat denaturation ran as a single band detectable by protein staining.

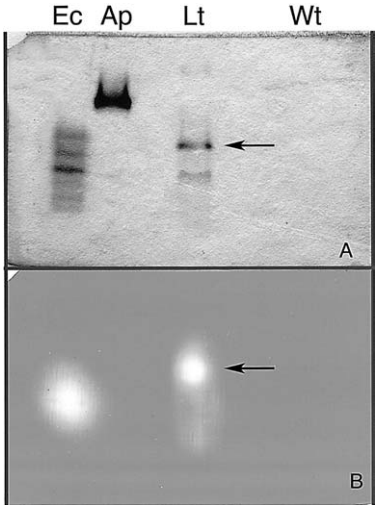

Fig. 1. Activity staining of MCOs separated using SDS-PAGE. For phenoloxidase staining with 1,8-diaminonaphthalene (A), lanes Ec and Ap were loaded with equivalent amounts of ABTS-oxidizing activity from the *yac*K protein and Aplacc1.1 enzyme pools, respectively. Lane Lt was loaded with 200 μg total protein from partially purified extracts of suspension-cultured tobacco cells transformed with the Lt*lacc2.2* gene, while lane Wt was loaded with 200 μg total protein prepared in an identical fashion from tobacco cells transformed with the control vector. For detecting ferroxidase activity (B), the amount loaded for each sample was 5× that loaded for phenoloxidase staining. Arrows denote the position of the transgenic yellow-poplar enzyme. For reference, the sycamore maple and *E. coli* LMCO proteins migrate with apparent molecular masses of 97.4 and 54 kDa, respectively, in denaturing SDS-PAGE gels [20,34].

2.3. Structural modeling of laccase

ClustalW alignment of amino acids encoded by Ap*lacc1.1*, Lt*lacc2.2* and part of the yeast Fet3 protein (truncated to remove the membrane spanning domain that is absent from the other LMCOs) illustrated the high degree of homology between these proteins (Fig. 2). Comparison of the amino acid residues between the two plant MCOs showed 41% identity and 60% similarity, while comparisons of either plant sequence with the yeast sequence found about 20% identity and 35% similarity. A similar alignment of the Lt-lacc2.2 protein with a laccase from *Coprinus cinereus*, for which a crystal structure is available [11], was used to generate the structural model shown in Fig. 3.

3. Discussion

This is the first report of ferroxidase activity associated with an LMCO from higher plants. Two independent studies recently reported that a ceruloplasmin-like MCO is involved with high-affinity iron uptake in the green alga, *Chlamydomonas reinhardtii* [16,24]. Thus, it may well be that MCO-dependent iron uptake systems are as broadly distributed across the plant kingdom as they are across other eukaryotes.

From the time that the first plant LMCO gene was cloned [22], sequence comparisons, such as that depicted in Fig. 2, have suggested it would be difficult to distinguish LMCOs whose primary physiological function was to oxidize phenolic substrates from those that oxidize iron or other metals. Such distinctions would be impossible to draw using plant extracts that contain multiple LMCO isozymes. Although previous attempts in this lab to demonstrate ferroxidase activity in the plant LMCOs from *A. pseudoplatanus* and *R. vernicifera* were unsuccessful, Fig. 1 clearly shows that both phenoloxidase and ferroxidase activities are associated with the Lt*lacc2.2* gene product. Thus, plant LMCOs are capable of catalyzing the same range of enzyme activities noted for MCOs in other organisms.

Unlike the sycamore maple (Aplacc1.1) enzyme, which was secreted by transgenic tobacco cells [8], yellow-poplar and *Zinnia elegans* LMCOs expressed in transgenic tobacco cells were not released by protoplasting, but only by complete cell rupture (Dean, unpublished data). Since the yellow-poplar LMCO was fully soluble in cell lysates, the enzyme

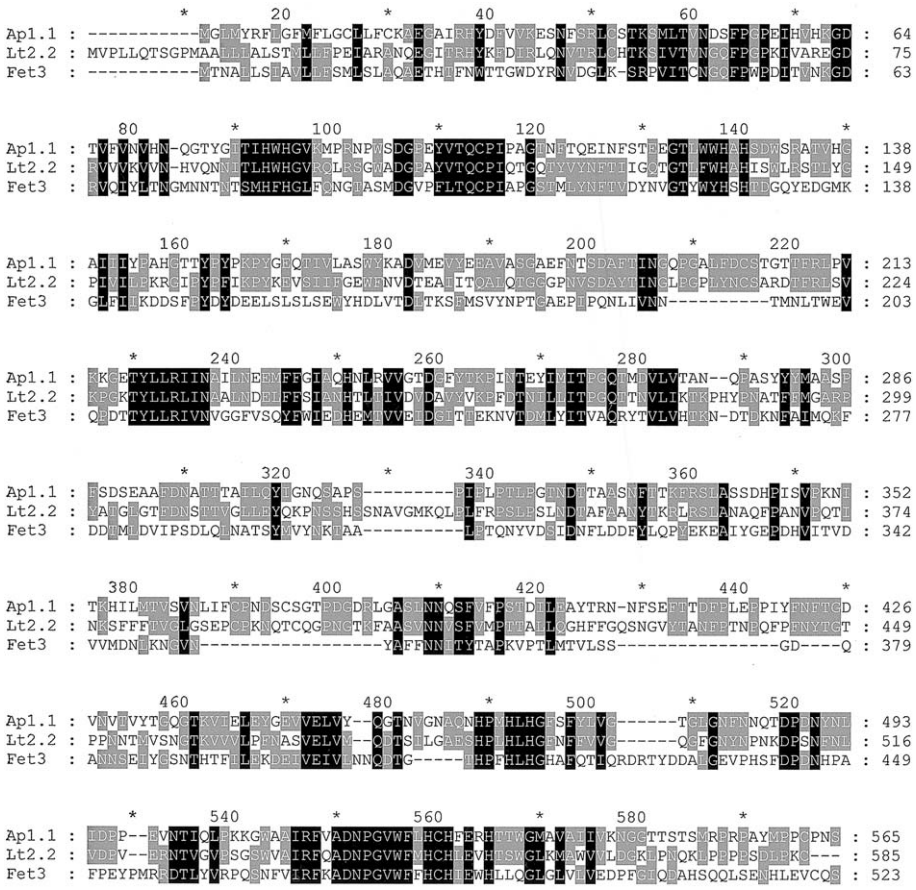

Fig. 2. Amino acid sequences of two plant laccases aligned with the yeast Fet3 protein. ClustalW was used to align the complete protein sequences of the Ap*lacc1.1* and Lt*lacc2.2* gene products, as well as a Fet3 protein sequence from which the carboxyl-terminal 113 amino acid transmembrane domain had been deleted. Amino acids shaded in black are strongly conserved between all three proteins, whereas gray shading indicates conserved residues shared by only two proteins.

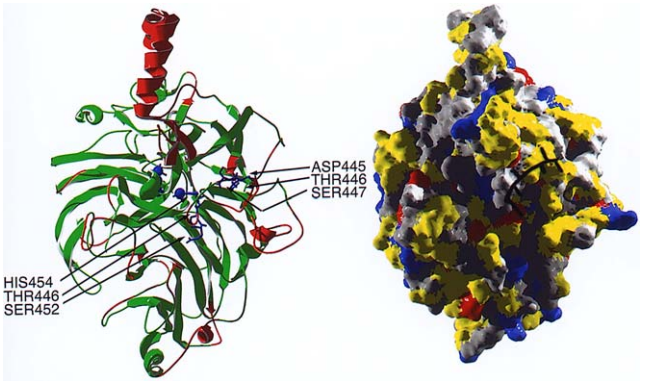

Fig. 3. Structural models for the Ltlacc2.2 LMCO protein. A strand representation of the model noting critical amino acid residues near the Type-1 copper center is depicted on the left. The panel on the right shows a molecular surface of the model with neutral, polar, acid, and basic amino acids represented by gray, yellow, red and blue, respectively. The curved black line indicates the approximate position of the residues labeled in the strand representation.

must have been retained in an intracellular compartment of the transgenic tobacco cells. If this intracellular localization results in a Ltlacc2.2 enzyme having little or no glycosylation, it could help explain the observation that in SDS-PAGE gels the Ltlacc2.2 enzyme had a migration rate closer to the *E. coli yac*K MCO than the Aplacc1.1 enzyme (Fig. 1). The mature peptides of these three enzymes are roughly equivalent in size (552, 488, and 542 amino acids for the yellow-poplar, *E. coli* and sycamore maple enzymes, respectively). The *E. coli* enzyme has a single potential glycosylation sites and is not glycosylated, while the sycamore maple LMCO has 17 potential glycosylation sites and is highly glycosylated [22,35]. The yellow-poplar LMCO sequence contains 14 putative glycosylation sites, but the amount of purified enzyme was insufficient to determine the glycosyl content of the heterologously expressed protein. It should be noted, however, that the zymogram gel system used in this study does not heat-denature proteins before running and the migration rates of proteins in such a gel format cannot be strictly compared. As these observations were made with transgenic enzyme expressed heterologously in tobacco cells, it cannot be said for certain whether or not the yellow-poplar LMCO is normally localized intracellularly, or whether it is highly glycosylated when expressed in yellow-poplar xylem.

Sequence alignment and modeling of the Ltlacc2.2 LMCO identified a region of the protein that may be influential in conferring ferroxidase activity to these enzymes

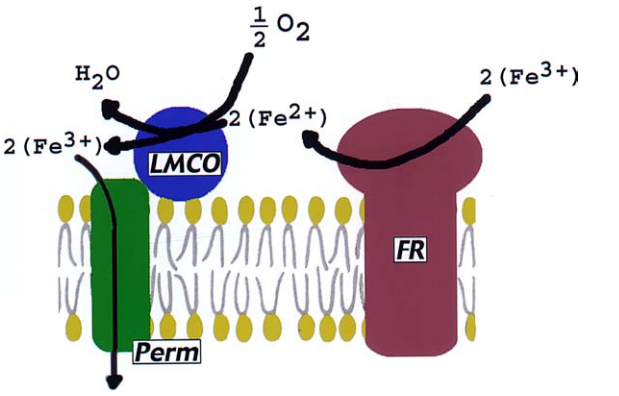

Fig. 4. Generalized model for an LMCO-dependent iron uptake system in plants. In a system analogous to that described for *S. cerevisiae* [33], iron reduced to its ferrous state via the action of ferrireductases (FR) [31], is oxidized back to its trivalent, ferric form by the action of an LMCO. The marginally soluble ferric ion is then immediately passed through the membrane with assistance from a trivalent metal-specific, LMCO-associated permease (Perm).

(Fig. 3). The loop containing residues 476–486 in the Ltlacc2.2 sequence is highly homologous to a region in the yeast Fet3 MCO (residues 409–414) that modeling has identified as a potential interaction site for iron (Hoopes and Dean, unpublished). Specifically, Fe^{2+} may be interacting specifically with an aspartyl residue in the sequence QDT*H, where the H is a liganding residue for the Type-1 Cu center and asterisks represent a short, variable stretch of amino acids. This residue is a target for site-directed mutagenesis studies to understand how these enzymes discriminate between substrates.

The possibility that plant LMCOs could function as components of iron uptake pumps provides an alternative rationale for their strong expression in lignifying vascular tissues (Fig. 4). Such pumps may be required in these tissues to reduce extracellular free iron to levels where it is not readily available to interact with the H_2O_2 that must be generated for peroxidase-catalyzed monolignol coupling [13]. The (Fenton) reaction between Fe^{2+} and H_2O_2 generates highly toxic ROS, OH· and OH^-, particularly when reducing agents, such as ascorbate, are available to cycle iron back to Fe^{2+}. These radicals can cause severe damage to membranes, proteins and DNA, and given the close proximity of dividing meristematic cells in the cambium to lignifying cells, it seems reasonable that there should be mechanisms to keep free iron levels very low in these tissues.

The involvement of vascular tissue LMCOs in a fundamental metabolic process, such as iron metabolism, would mean that disrupting their expression via gene manipulation could lead to a wide variety of phenotypic outcomes in transgenic plants. Disrupted expression of LMCOs involved in iron metabolism could impact lignin levels by affecting heme biosynthesis and peroxidase assembly. However, overall poor growth and leaf chlorosis, as is often observed in plants grown under iron deficient conditions, could be expected in knockout mutants of such LMCOs. Such a phenotype has been seen in at least one antisense laccase mutant generated in *Arabidopsis thaliana* (Halpin, personal communication). Further studies will be required to demonstrate a direct link between plant LMCOs and iron metabolism.

4. Methods

4.1. Tissue-culture

Preparation of the plant expression vector in which the Lt*lacc2.2* LMCO cDNA was placed under the control of a dual-enhanced CaMV 35S promoter was described previously [23]. Transformations of *Nicotiana tabacum* L. Cv. Bright Yellow 2 (BY2) cells by micro-projectile bombardment using either the Lt*lacc2.2* expression construct or the empty vector (pKYLX80) control were performed as previously described [23]. For production of transgenic protein, suspension cultures of tobacco cell lines transformed with the LMCO expression construct or the empty vector control were grown in 2.8 l Fernbach flasks containing 500 ml of medium. The growth medium consisted of a commercial preparation of Murashige-Skoog salts (Life Technologies, Bethesada, MD) containing 88 mM sucrose, 2.7 mM potassium monophosphate, 343 μM kanamycin sulfate, 156 μM *myo*-inositol, 3 μM thiamine HCl, and 0.8 μM 2,4-D. The medium pH was adjusted to 5.7, and cells were grown at 24 °C in the dark with shaking at 110 rpm. The lines were subcultured every 7 days using a 1/10 (v/v) inoculation into fresh medium. Cells for enzyme extraction were harvested 7 days after transfer to fresh medium.

4.2. Enzymes and reagents

The laccase-like MCO from *A. pseudoplatanus* (Ap*lacc1.1*) was purified from suspension-cultured cells as previously described [38]. The Ltlacc2.2 LMCO was concentrated and partially purified as follows. Suspension-cultured cells (900 g) were harvested by filtration and resuspended (1.6 g ml^{-1}) in chilled extraction buffer consisting of 50 mM sodium acetate (pH 6.0), 10 mM NaCl, 1 mM $CuSO_4$, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10% (v/v) glycerol. Cells were homogenized, and insoluble debris was removed by centrifugation at 4 °C and 20,000 × *g* for 30 min. The supernatant was filtered through Miracloth (Calbiochem-Novabiochem Corp., San Diego, CA) and brought to 40% saturation with slow addition of solid ammonium sulfate. The solution was stirred overnight at 4 °C, and insoluble material was removed by centrifugation at 4 °C and 20,000 × *g* for 30 min. The supernatant was filtered through Miracloth, mixed with an equivalent volume of water, and PMSF was added to a final concentration of 1 mM. The enzyme pool was then concentrated 140-fold against a 10 kDa molecular mass cutoff ultrafiltration membrane, and stored at –80 °C until use. Purification of the *E. coli yac*K gene product, an MCO having ferroxidase activity, was as described elsewhere [20]. All other chemicals and reagents

used in this study were of the highest purity commercially available and were used without further purification.

4.3. Protein and enzyme assays

Protein was quantified using the ELS protein assay kit (Boehringer Mannheim, Indianapolis, IN) against BSA standards. Phenoloxidase activity for each LMCO was determined from the increase in absorbance at 420 nm due to the oxidation of 5 mM 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) in 100 mM sodium acetate buffer (pH 5.0). The molar absorptivity of oxidized ABTS (ε_{420}) was taken as 36 mM^{-1} cm^{-1}. Ferroxidase activity was determined using ferrous sulfate as the electron donor and 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine) as a specific chelator to bind ferrous iron remaining at the end of the reaction. Reactions were carried out in disposable cuvettes containing 100 μM ferrous sulfate in 100 mM sodium acetate buffer (pH 5.0). The reactions were quenched by addition of ferrozine to a final concentration of 1.5 mM, and the rate of Fe^{2+} oxidation was calculated from the decreased absorbance at 560 nm using a molar absorptivity of e_{560}= 25,400 M^{-1} cm^{-1} for the Fe^{2+}–ferrozine complex.

4.4. Gel electrophoresis

SDS-PAGE based zymograms were performed using gels prepared according to the protocol of Laemmli [21]. Aliquots of recombinant laccases, loaded on the basis of equal amounts of phenoloxidase activity, were mixed 1/1 (v/v) with 2× Laemmli sample buffer containing no reducing agents, and loaded directly onto gels without heat denaturation. After electrophoresis, gels were stained for phenoloxidase activity using 1,8-diaminonapthalene as described previously [18]. Ferroxidase zymograms were performed as described by Yuan et al. [39] with modifications as follows. Ferroxidase activity was detected by soaking the gel in 100 mM sodium acetate buffer (pH 5.0) containing 5% (v/v) glycerol, and 1 mM $CuSO_4$ for 1 h. Gels were subsequently washed in 100 mM sodium acetate buffer (pH 5.0) containing 0.2 mM $FeSO_4$ for 1 h. Gels were then washed twice with deionized water to remove surface iron, and incubated at room temperature for 8 h in a dark humidifying chamber. Cleared zones representing ferroxidase activity were visualized by applying 1.5 mM ferrozine in water dropwise to the surface of the gel. Gels developed in approximately 10 min and were immediately documented using an Alpha Innotech ChemImager 4000 imaging system and AlphaEase software (Alpha Innotech Corp., San Leandro, CA).

4.5. Sequence analysis and modeling

The mature sequences of the Aplacc1.1 (GenBank U12757), Ltlacc2.2 (GenBank U73104), and the *S. cerevisiae* Fet3 (GenBank L25090) proteins were aligned using the online ClustalW tool [17] available through the Baylor College of Medicine server (dot.imgen.bcm.tmc.edu:9331/multi-align/Options/clustalw.html). The membrane-anchoring domain (115 amino acids) at the carboxyl-terminus of the Fet3 protein was removed prior to alignment. Box-shading to highlight conserved residues, as shown in Fig. 2, was performed using GeneDoc, ver 2.5 (www.psc.edu/biomed/genedoc/). The mature protein sequence of Ltlacc2.2 was aligned to the protein sequence of the laccase from *C. cinereus*, for which a crystal structure has been reported [11], using the GAP alignment function of the GCG Wisconsin Package, ver. 10.2 (Pharmacopeia, Inc., Burlington, MA). The GAP initiation penalty was set at 12, but all other parameters were maintained at default values. Variations in gap elongation penalty did not seem to significantly alter the alignments. The resulting alignment then used to generate a draft structural model for Ltlacc2.2 using the SWISS-MODEL server accessible through the Swiss-Pdb Viewer (www.expasy.ch/spdbv/). The resulting model was hand-refined at failure points, based on minimization of threading energy and number of identical residues, and resubmitted until a stable full-length model was identified.

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ORIGINAL ARTICLE

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Gene structure and molecular analysis of the laccase-like multicopper oxidase (LMCO) gene family in *Arabidopsis thaliana*

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Abstract Completed genome sequences have made it clear that multicopper oxidases related to laccase are widely distributed as multigene families in higher plants. Laccase-like multicopper oxidase (LMCO) sequences culled from GenBank and the *Arabidopsis thaliana* genome, as well as those from several newly cloned genes, were used to construct a gene phylogeny that clearly divided plant LMCOs into six distinct classes, at least three of which predate the evolutionary divergence of angiosperms and gymnosperms. Alignments of the predicted amino acid sequences highlighted regions of variable sequence flanked by the highly conserved copper-binding domains that characterize members of this enzyme family. All of the predicted proteins contained apparent signal sequences. The expression of 13 of the 17 LMCO genes in *A. thaliana* was assessed in different tissues at various stages of development using RT-PCR. A diversity of expression patterns was demonstrated with some genes being expressed in a constitutive fashion, while others were only expressed in specific tissues at a particular stage of development. Only a few of the LMCO genes were expressed in a pattern that could be considered consistent with a major role for these enzymes in lignin deposition. These results are discussed in the context of other potential physiological functions for plant LMCOs, such as iron metabolism and wound healing.

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Abbreviations BLAST: Basic local alignment search tool · CTAB: Cetyl trimethylammonium bromide · LMCO: Laccase-like multicopper oxidase · MCO: Multicopper oxidase · MPSS: Massively parallel signature sequencing · PCR: Polymerase chain reaction · pI: Isoelectric point · RACE: Rapid amplification of cDNA ends · RT-PCR: Reverse transcript polymerase chain reaction · TBE: Tris borate EDTA buffer

Introduction

Multicopper oxidases (MCOs) constitute an enzyme superfamily that includes plant and fungal laccases (Mayer and Staples 2002), yeast ferroxidases (De Silva et al. 1995) bacterial metal oxidases (Brouwers et al. 1999; Grass and Rensing 2001), mammalian and avian ceruloplasmin (Musci 2001), and plant ascorbate oxidases (Messerschmidt 1997). The so-called ''laccases'' are a particularly disparate subgroup of MCOs that cross bacterial, fungal and plant taxonomic categories. The physiological functions proposed for these enzymes in different fungal species extend from lignin degradation (Eggert et al. 1997), to pigment production (Aramayo and Timberlake 1990) and pathogenic virulence (Salas et al. 1996), while bacterial ''laccases'' have been implicated in metal transport and homeostasis (Brouwers et al. 1999; Grass and Rensing 2001; Kim et al. 2001), and plant laccases have been proposed to assist in wound healing and lignin biosynthesis (Butt 1980; Dean and Eriksson 1994). Although some enzymes in the MCO family oxidize specific, identified substrates, and have been named accordingly, most of the better known MCOs, including the ''laccases,'' are capable of oxidizing multiple potential substrates, and this has created significant confusion in enzyme nomenclature (Mayer

and Harel 1979). Strictly speaking, the name "laccase" should be reserved for those members of the MCO family that are found in plant saps containing the unsaturated alkylcatechols ("laccol," also known as "urushiol") that are their natural substrates (Du et al. 1984). However, the name has frequently been used to identify any monomeric MCO that contains four copper atoms in a specific arrangement of Type-1, Type-2 and Type-3 electromagnetic centers and displays some level of phenoloxidase activity (Messerschmidt 1997). Recognizing the multiplicity of functions likely performed by the numerous MCOs appearing in plant genomes, we recently elected to abandon our previous use of "laccase" to cover all monomeric, four-copper MCOs in favor of "laccase-like multicopper oxidases" or "LMCOs" (Hoopes and Dean 2004). Following the traditions of enzyme nomenclature, we presume that more descriptive names will be assigned once specific catalytic reactions and metabolic functions are defined for the various LMCOs.

The precise physiological function(s) of LMCOs in plants is unclear, due in no small part to the size and complexity of the gene families examined in a variety of plant species. The enzyme has been purified from the differentiating xylem of *Acer pseudoplatanus*, *Pinus taeda*, *Zinnia elegans* and *Liriodendron tulipifera* (Sterjiades et al. 1992; Bao et al. 1993; Liu et al. 1994; LaFayette et al. 1999). The enzyme secreted by *A. pseudoplatanus* cells grown in suspension culture was shown to oxidize lignin monomers in vitro (Sterjiades et al. 1992) suggesting that it had the capacity to be involved in lignification. The prototype LMCO, the laccase isolated from *R. vernicifera* was long ago purported to play a role in wound healing as part of an herbivore or pathogen defense response (Hüttermann et al. 2001; Mayer and Staples 2002). When the stem of *Rhus vernicifera* (Japanese lacquer tree) is cut, laccase initiates an oxidative polymerization reaction with the alkylcatechols in the latex sap, producing a hard, protective seal over the wound (Messerschmidt 1997). Most recently, we have presented biochemical evidence that could support a role for plant LMCOs in iron metabolism (Hoopes and Dean 2004).

Given their potential to catalyze multiple, divergent oxidative reactions, efforts to resolve the physiological function(s) of plant LMCOs would be helped by better delineation of the size and complexity of this gene family in a model plant system. There have been conflicting reports in the literature about how widespread LMCOs are across the plant kingdom (Mayer and Staples 2002), and few studies have analyzed the age and divergence of the gene family within plants. In the study reported here, the LMCO gene family was characterized by phylogenetic analysis across a broad range of species, and patterns of gene-specific expression within a reference species, *Arabidopsis thaliana*, are described. Access to the complete *Arabidopsis* genome sequence facilitated design of gene-specific primers that were used to study gene expression patterns in different organs during development. By comparing the phylogenetic history and differential gene expression of the LMCO gene family, we demonstrate that LMCOs are broadly distributed across higher plant orders, have evolved into six highly divergent phylogenetic groups that comprise both ancient and recent gene duplications, and are expressed throughout the plant during development. Most of the phylogenetic groups appear to have evolved prior to the divergence of angiosperms and gymnosperms, and within *Arabidopsis*, each phylogenetic group includes LMCO genes expressed constitutively or in developmentally regulated patterns. Sequence homology alignments highlight regions of structural divergence that may be of importance in defining functional differences between LMCO gene family members expressed simultaneously in the same cells and tissues.

Materials and methods

Collection of laccase sequences

To identify additional putative plant LMCO sequences in GenBank, a BLAST search was performed using the genes previously characterized from *L. tulipifera* (LTU73104) and *A. pseudoplatanus* (APU12757). The 44 sequences obtained from *A. thaliana*, *L. tulipifera*, *Nicotiana tabacum*, *P. taeda*, *P. trichocarpa*, *R. vernicifera* and *O. sativa* are shown in Table 1.

Initial comparisons of these genes revealed multiple divergent classes of plant LMCOs. To recover LMCO genes from additional species, degenerate oligonucleotide primers (Table 2) were used to amplify genomic DNA using PCR. Multiple primer combinations were used to increase the likelihood of isolating genes from each of the major phylogenetic classes. Genomic DNA extracted from the dicot angiosperms, *A. pseudoplatanus* (sycamore maple), *B. napus* (canola), and *A. procurrens* (rock cress), as well as the gymnosperms, *P. taeda* (loblolly pine) and *G. biloba*, using a modified CTAB protocol (Stewart and Via 1993), served as template for these reactions. The amplification reactions (50 µl) contained approximately 0.8 µg genomic DNA, 2.5 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), 5 µl AmpliTaq Gold reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs and 0.2 µM each of forward and reverse primer. The amplification protocol included an initial 10 min denaturation at 94°C, 40 cycles of 94°C for 1 min, 49°C for 1 min and 72°C for 2 min, and a final 72°C extension for 7 min. Reaction products were resolved on 0.8% agarose gels in 1X TBE buffer. Amplified DNA was cloned into the pCR II-Topo vector (Invitrogen, Carlsbad, CA, USA) and sequenced. Single-pass DNA sequence was used to identify the exons in each amplimer, and these were sequenced redundantly on each strand. Exon sequences used to assemble putative amino acid sequences for the phylogenetic analyses were deposited separately as linked exon files in GenBank.

Table 1 Accession numbers and encoded protein information for LMCO sequences from GenBank used to construct the phylogeny

Gene name	Accession no.	Species name	Full length	Amino acids
Acer 1	APU12757	*A. pseudoplatanus*	Yes	565
At1g18140	AAF78389	*A. thaliana*	Yes	581
At2g29130	AC005315	*A. thaliana*	Yes	573
At2g30210	AC004165	*A. thaliana*	Yes	570
At2g38080	AC003028	*A. thaliana*	Yes	554
At2g40370	AC007020	*A. thaliana*	Yes	580
At2g46570	AC006418	*A. thaliana*	Yes	569
At3g09220	AB005240	*A. thaliana*	Yes	567
At5g01040	AL137189	*A. thaliana*	Yes	584
At5g01050	AL137189	*A. thaliana*	Yes	586
At5g01190	AL137189	*A. thaliana*	Yes	553
At5g03260	AB005240	*A. thaliana*	Yes	555
At5g05390	AB010692	*A. thaliana*	Yes	565
At5g07130	AL163652	*A. thaliana*	Yes	569
At5g09360	AL391712	*A. thaliana*	Yes	569
At5g48100	AB017064	*A. thaliana*	Yes	565
At5g58910	AB016885	*A. thaliana*	Yes	566
At5g60020	AB015475	*A. thaliana*	Yes	577
Liriodendron 1	LTU73103	*L. tulipifera*	Yes	570
Liriodendron 2	LTU73104	*L. tulipifera*	Yes	585
Liriodendron 3	LTU73105	*L. tulipifera*	Yes	586
Liriodendron 4	LTU73106	*L. tulipifera*	Yes	585
Nicotiana 1	NTU43542	*N. tabacum*	No	409
Nicotiana 3	NTU45243	*N. tabacum*	Yes	557
Oryza 1	AF047697	*O. sativa*	Yes	551
Oryza 2	BAB92844	*O. sativa*	No?	520
Oryza 3	BAB92845	*O. sativa*	Yes	554
Oryza 4	BAB92843	*O. sativa*	Yes	586
Oryza 5	BAB90733	*O. sativa*	Yes	562
Oryza 6	BAB86465	*O. sativa*	Yes	563
Oryza 7	BAB86452	*O. sativa*	Yes	577
Pinus 1	AF132119	*P. taeda*	Yes	586
Pinus 2	AF132120	*P. taeda*	Yes	576
Pinus 3	AF132121	*P. taeda*	Yes	574
Pinus 4	AF132122	*P. taeda*	Yes	570
Pinus 5	AF132123	*P. taeda*	Yes	591
Pinus 6	AF132124	*P. taeda*	Yes	578
Pinus 7	AF132125	*P. taeda*	Yes	555
Pinus 8	AF132126	*P. taeda*	Yes	577
Populus 1	PTY13769	*P. trichocarpa*	No	407
Populus 2	PTY13770	*P. trichocarpa*	No	437
Populus 3	PTY13771	*P. trichocarpa*	Yes	555
Populus 90	PTY13772	*P. trichocarpa*	Yes	574
Populus 110	PTY13773	*P. trichocarpa*	Yes	580
Rhus 1	BAB63411	*R. verniciflera*	No	533

Sequence analysis

Non-coding DNA sequences were identified through GenBank annotations, intron prediction software (NetPlantGene, http://www.cbs.dtu.dk/services/NetPGene/) (Hebsgaard et al. 1996) and by sequence alignment and translation, and non-coding regions were removed from nucleotide sequences using the GCG Suite of software tools (Wisconsin Package version 10.2, Genetics Computer Group [GCG], Madison, WI, USA). Putative N-terminal signal peptides were identified using the SignalP server (http://www.cbs.dtu.dk/services/SignalP/) (Nielsen et al. 1997) and were tested for likely subcellular targeting using TargetP version 1.01(http://www.cbs.dtu.dk/services/TargetP/). In the latter system subcellular targeting is predicted based on sequence similarity to known N-terminal chloroplast transit peptides, mitochondrial targeting peptides or secretory pathway signal peptides (Emanuelsson et al. 2000). Due to the high level of sequence divergence in the signal peptides, they were removed prior to further analyses. Thus, mature amino acid sequences were aligned in GCG using default parameters in PILEUP, and the resulting alignments were optimized manually.

Gene genealogies were estimated using PAUP 4.0*, version 4.0b10 (PPC) (Swofford 2000). Amino acid mean character distances were used to construct neighbor-joining trees, and 100 bootstrap replicates were run to test for degree of phylogenetic confidence. To determine whether similar tree topologies could be obtained from different phylogenetic algorithms, a strict consensus gene phylogeny was constructed from a maximum parsimony heuristic search.

Table 2 Degenerate oligonucleotide primers used to amplify unknown LMCOs representing different protein groups from divergent plant species

Primer name	Primer sequence	Location
LMCO_2F1	5′ KAY GCW MGR GAA GGN GAY A 3′	Exon 2
LMCO_3F1	5′ CAA TGY CCR ATY MGA CCN G 3′	Exon 3
LMCO_4F1	5′ GAA TGG TGG AAR KCN GAY 3′	Exon 4
LMCO_4F2	5′ GTC AAC CNG GNG ATC TYT A 3′	Exon 4
LMCO_5R1	5′ ATG TAG AAN TCG TAN CCR TG 3′	Exon 5
LMCO_6R1	5′ ART CCC CAH GTN GTR TGY A 3′	Exon 6

The mixed base codes, as per the International Union of Biochemistry (*IUB*), represent A, C or T (*H*), Keto (*K*), amino-A or -C (*M*), any nucleotide (*N*), purine (*R*), pyrimidine (*Y*), and A or T (*W*)

To compare the average level of sequence similarity along the length of the laccase protein, PLOTSIMILARITY (window size = 100) from the GCG Suite was run using all available full-length aligned sequences, with signal peptides included. The analysis was also performed separately for each phylogenetic group of plant LMCOs (window size = 50).

The theoretical isoelectric focusing point (pI) was calculated for full-length sequences, minus the signal peptides, using the ProtParam tool on the ExPASy proteomics server (Appel et al. 1994) (http://www.expasy.ch/tools/protparam.html). The average pI was determined for each phylogenetic group, and a Bonferroni/Dunn test from StatView version 5.0.1 was used to test for significant differences in average pI between groups.

RNA isolation and cDNA synthesis

For RNA extraction, tissue was collected from 2-week-old seedlings grown in liquid medium and from 6-week-old adult plants grown in soil. Wild-type *A. thaliana* (var. Columbia) seeds were sterilized in 50% ethanol for 1 min, transferred to 2.5% sodium hypochlorite, 0.1% Tween-20 for 10 min, and washed three times in sterile water. For seedling tissue, seeds were germinated and grown for 2 weeks in liquid Murashige and Skoog medium (Invitrogen Life Technologies, Carlsbad, CA, USA) containing sucrose (20 g/l), shaking at 22 °C under constant illumination. Seedling leaves and roots were separated, flash-frozen in liquid nitrogen, and stored at −80°C until needed. For mature tissues, seeds were sown in pots containing potting soil and grown at 22°C under 16-h illumination. Soil was washed from the roots, and tissue was collected from whole roots, from rosette leaves at varying developmental stages (= 1, 1.5−2, and 3−4 cm long), from separate inflorescence stem internodes (first, second, third and fourth internodes from the base of the plant, and the terminal internode, which consisted of only the top 0.5−1.0 cm of the inflorescence stem), from fully closed flower buds (petals not visible), and from open flower buds (siliques not exerted). All tissues were flash frozen in liquid nitrogen and stored at −80°C.

Approximately 2 g of tissue were homogenized with a mortar and pestle in liquid nitrogen. After grinding, 6 ml NETS buffer (100 mM NaCl, 10 mM Tris/HCl pH 7.5, 1 mM EDTA, 1% SDS), 2 ml phenol, 2 ml chloroform/isoamyl alcohol (24:1), and 100 µl β-mercaptoethanol were added, and the homogenized material was centrifuged for 15 min at 7,800×*g*. The aqueous phase was extracted twice with chloroform/isoamyl alcohol, and RNA was precipitated by addition of LiCl to 4 M final concentration and incubation overnight at 4°C (Sambrook et al. 1989). The RNA was pelleted by centrifugation and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). The RNA was subsequently re-precipitated by the addition of 1/10th volume 3 M sodium acetate, pH 4.8, and two volumes of ethanol prior to overnight incubation at −20°C. After pelleting by centrifugation, the RNA was washed in cold 70% (v:v) aqueous ethanol, resuspended in water, and treated with DNAse I following the provided protocol (Ambion, Inc., Austin, TX, USA). Final RNA concentrations were determined by UV absorbance.

Total RNA (5 µg) was used as template for reverse transcription into cDNA in the 3′ RACE System using the adapter primer and protocols supplied by the manufacturer (Invitrogen Life Technologies). To quantify the cDNA yield, 2 µl of product from each reaction was diluted with TE to a total volume of 100 µl. PicoGreen solution (Molecular Probes, Eugene, OR, USA) was diluted 1:200 with TE, and an equal volume was added to the diluted cDNA. After incubating for five minutes, the DNA concentration was measured against lambda DNA standards ranging in concentration from 0 ng/ml to 1,000 ng/ml using a Biolumin 960 spectrophotometer (Molecular Dynamics, Sunnyvale, CA, USA).

Expression analyses

Genomic DNA sequences for thirteen *A. thaliana* LMCOs, including approximately 1,000 bases upstream of the start codon and 300 bases downstream of the stop codon, were obtained from GenBank. Since northern blot analysis showed that most of the genes were expressed at nearly undetectable levels, titrated RT-PCR was used to compare relative levels of expression between genes and between tissue types. For each gene, exact match oligonucleotide primers (Table 3) were designed to span the 3′-terminal intron and extend 30 to 250 nucleotides into the 3′-UTR downstream of the stop codon. This design simultaneously provided gene-specificity and an ability to test for potential contamination from genomic DNA. For comparison, control reactions containing genomic DNA were run for each set of primers. All primers were

Table 3 Gene-specific oligonucleotide primers used to amplify 3′UTR sequences from *Arabidopsis* LMCOs

Primer name	Primer sequence
2g29130_1559F	5′ GGGTCGCCATCCGCTTCCTTGCCGATAAT 3′
2g29130_1845R	5′ CCATCGTTTTTCAGTTCACTCTTTATACAA 3′
2g30210_1545F	5′ TGGTTGGGTGGCAATTCGGTTCGTCGCTGA 3′
2g30210_1802R	5′ ATAGAGCAGTTGTCCATCTTTAACATATCA 3′
2g38080_1500F	5′ ATGGGTCGTCATCAGATTCAGAGCAGATAA 3′
2g38080_1888R	5′ CATATAAGAGGTGTGTTAGAGACAATAATA 3′
2g40370_1544F	5′ CTCTCAGAAATACTGTCGGTGTACCTGTTA 3′
2g40370_1773R	5′ TTGAATAGAAGAACATGAAAAACTATAATG 3′
2g46570_1536F	5′ AGTAGGAGGTTGGGCGGCAATTCGGTTTGT 3′
2g46570_1844R	5′ ATACCATAGTCTCTATCCCTTTGAATGACA 3′
3g09220_1503F	5′ GCAATCCCGTAACACTTTGGCTGTCCCTGT 3′
3g09220_1806R	5′ TGGACTACAATACAATTTCTTTACCGACTA 3′
5g01040_1542F	5′ ATGGGTTGTCCTCAGATTTATCGCTAATA 3′
5g01040_1986R	5′ TGTCTCTCGTACAAATCAGTTCAAGCATT 3′
5g01050_1542F	5′ ATGGGTTGTCCTCAGATTTATCGCTAATA 3′
5g01050_1882R	5′ GTGTAGAGAAATGGAAGCATGGTCGTAAA 3′
5g03260_1466F	5′ CGCCTGAGAGAAACACCGTTGGAGTACCTA 3′
5g03260_1743R	5′ CAGACACAAATCAACAGAAGGGAAATACAA 3′
5g05390_1531F	5′ GGATGGGCTGTTATCAGATTCGTGGCAGAT 3′
5g05390_1806R	5′ TATCTCATCTGACAATCATCACATCACTTA 3′
5g48100_1519F	5′ AGGAATGGTTGGATCGCTATCAGATTCGTA 3′
5g48100_1698R	5′ ATAGATAAAAGCATTTGGTAACATTTAAT 3′
5g58910_1535F	5′ GTTGGACTGCCATAAGATTCATCGCCGACA 3′
5g58910_1870R	5′ AAAGAAATTGCCAAAACAGAAGAATAACGT 3′
5g60020_1560F	5′ ATCTGGTGGATGGGCTGCTATTCGATTCCT 3′
5g60020_1824R	5′ GACCTCATGTCAAACCGATAAATGGCCGAG 3′

gene-specific with the exception of the forward primer for the tandemly duplicated At5g01040 and At5g01050 genes. The nearly identical coding regions for these two genes necessitated use of the same forward primer for both. However, the reverse primers for these two genes were from variable regions of the 3′ UTR, and included small, gene-specific indels that conferred adequate specificity to differentiate the gene products.

Primers were tested in reactions containing 0.7 μg of *Arabidopsis* genomic DNA to optimize reaction parameters and ensure efficient amplification. The reaction mixtures consisted of 20 ng cDNA template, 5 μl reaction buffer (500 mM KCl, 100 mM Tris-HCl and 1.0% Triton X-100), 2 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM each of forward and reverse primer, and 2.5 units Taq (Promega, Madison, WI, USA) in a total volume of 50 μl. The reaction mixture underwent an initial 3-min denaturation at 94°C, then 45 cycles at 94°C (45 s), 47°C (45 s) and 72°C (1 min), and a final extension time of 7 min at 72°C. Amplification products (20 μl aliquots) were run on 1.2% agarose gels in 1X TBE buffer and stained with ethidium bromide. PCR products were scored for presence/absence of bands and, in the cases where bands were not saturated, relative band intensity. To ensure equal cDNA input between different tissues, cDNA was quantified by Picogreen analysis and control PCR reactions were run using degenerate *Arabidopsis* actin primers (5′ GAR AAR ATG ACN CAR ATN ATG TTY GAR ACN TT 3′ and 5′ TCY TTN CTN ATR TCN ACR TCR CAY TTC ATD AT 3′). The actin control reactions were amplified for 35 cycles at 94°C (45 s), 42°C (45 s) and 68°C (45 s).

Results

LMCO gene isolation using degenerate oligonucleotide primers

Polymerase chain reaction (PCR) amplification with degenerate oligonucleotide primers designed against highly conserved LMCO structural domains was used to isolate three partial LMCO genes from *Arabis procurrens* genomic DNA, and seven partial LMCO genes from *Brassica napus* DNA (Table 4). Two LMCO genes isolated from *P. taeda* genomic DNA, Loblolly 90 and Loblolly 100, were found to have corresponding cDNA sequences in GenBank (AF132122 and AF132120, respectively). Single genes were recovered from *A. pseudoplatanus* and *Ginkgo biloba* genomic DNA. Intron sequences in these genomic DNA amplimers, which were inferred from amino acid alignments with well-characterized plant LMCOs, were not fully sequenced, and consequently only the exon sequences were deposited in GenBank as linked sequences as listed in Table 4. The inferred amino acid sequences corresponding to these exons were used to provide additional resolution in the phylogenetic analyses.

Sequence alignment

There are 17 genes in the *Arabidopsis* genome that are readily recognizable as LMCOs (Table 1). As of November 2004 the GenBank annotations for two other genes, At5g07130 and At5g58910, contained errors with

Table 4 New LMCOs recovered by PCR using degenerate oligonucleotide primers

Species	Gene	Accession no.	Primer combination	Length (bp)
A. procurrens	Arabis 61	AY645915 AY645916 AY645917	4F1/5R1	673
A. procurrens	Arabis 151	AY645941 AY645942	3F2/5R1	1,151
A. procurrens	Arabis 154	AY645910 AY645911	4F2/5R1	784
B. napus	Brassica 15	AY645918 AY645919 AY645920	4F1/6R1	1,105
B. napus	Brassica 19	AY645926 AY645927 AY645928	4F1/6R1	1073
B. napus	Brassica 76	AY645929 AY645930 AY645931	3F1/5R1	906
B. napus	Brassica 77	AY645932 AY645933	3F1/5R1	885
B. napus	Brassica 177	AY645921 AY645922	4F2/5R1	871
B. napus	Brassica 179	AY645923	4F2/5R1	828
B. napus	Brassica 182	AY645924 AY645925	4F2/5R1	828
A. pseudoplatanus	Acer 2	AY645912 AY645913 AY645914	2F1/6R1	899
G. biloba	Ginkgo 130	AY645934 AY645935 AY645936	3F1/5R1	1,057
P. taeda	Pinus 90	AY645907 AY645908 AY645909	3F1/5R1	1,131
P. taeda	Pinus 100	AY645937 AY645938 AY645939	3F1/5R1	1,128

Gene fragments were initially amplified from genomic DNA, and intron positions were identified using the *NetPlantGene* server and by comparison to introns in other species. Accession numbers are for the exon sequences deposited in GenBank as linked sequences. Primer combination refers to degenerate primer pair used to amplify product. The reported length refers to the predicted coding nucleotide sequence of amplified product

respect to splice junctions; however, these were corrected for the amino acid sequences used in this study. In addition, there are at least two annotated models for At1g18140 with very different structures, and while the structure used for the analyses reported here (AAF97830) appears to be most consistent with the other *Arabidopsis* LMCOs, it remains an outlier of the gene family. In general, gene structure was conserved with respect to the numbers and positioning of introns, although intron length varied substantially. Overall, 14 out of 17 genes were comprised of six exons separated by five introns (Fig. 1). In all of the LMCO genes, the first intron fell just within the mature coding sequence where it served to neatly divide signal sequences from coding sequences. The second intron position was also conserved in all LMCO genes where it bifurcated the first conserved copper-binding domain, HWHG, falling between the tryptophan and second histidine residues. LMCO genes At1g18140 and At2g30210 lacked introns 5 and 4, respectively, while At5g07130 lacked introns 3 and 5.

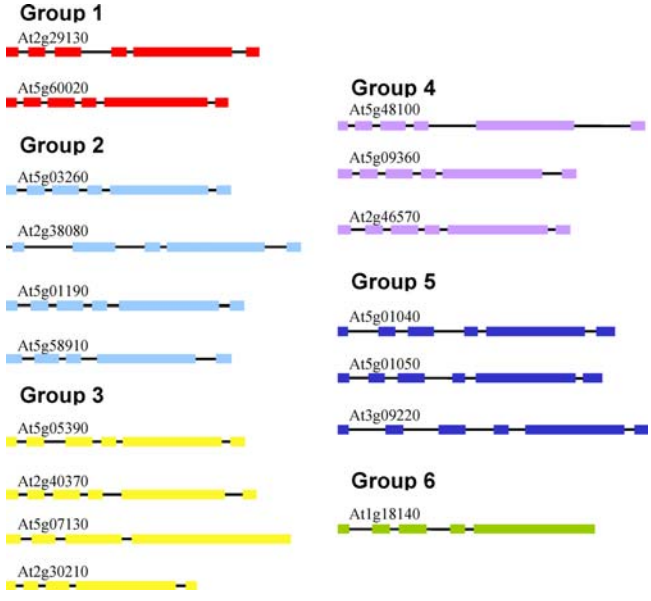
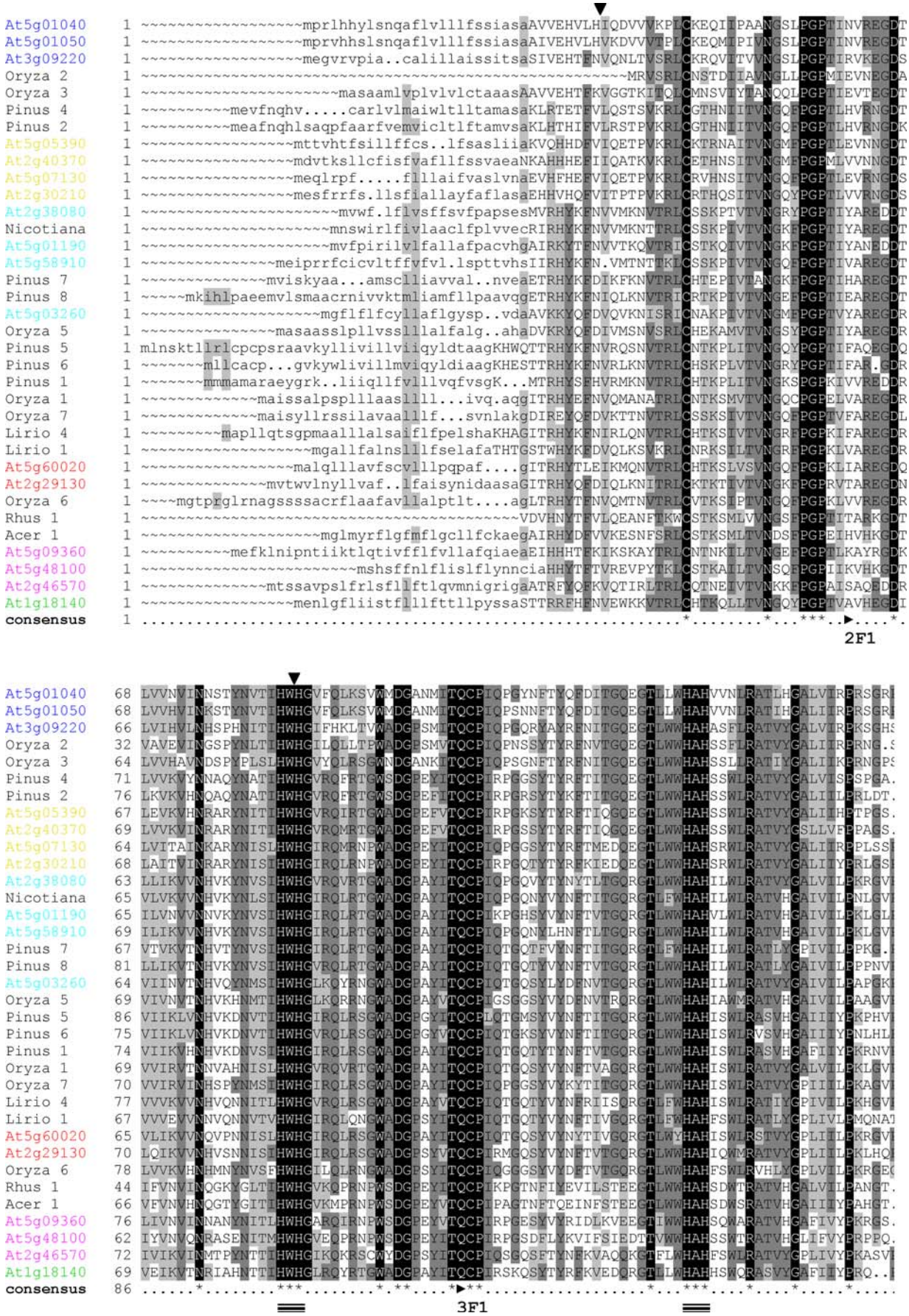
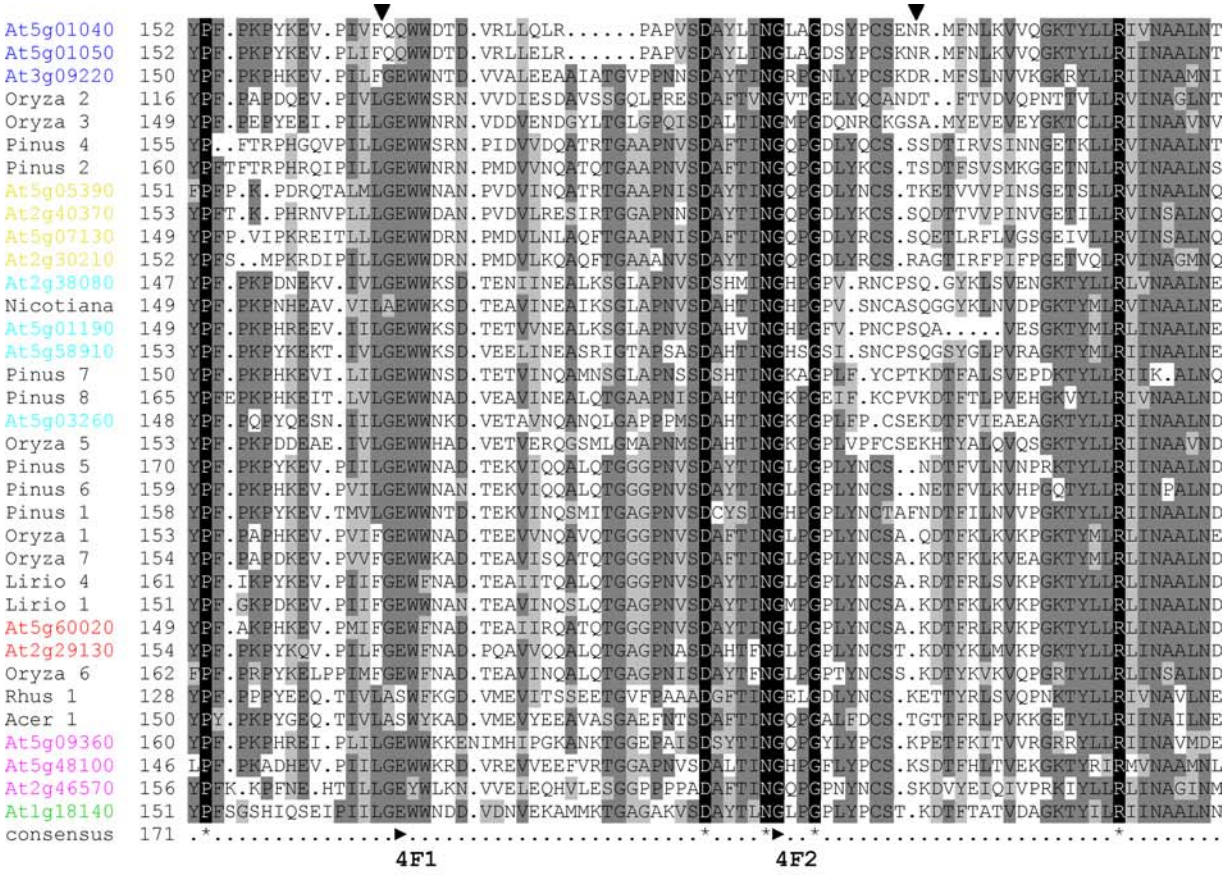
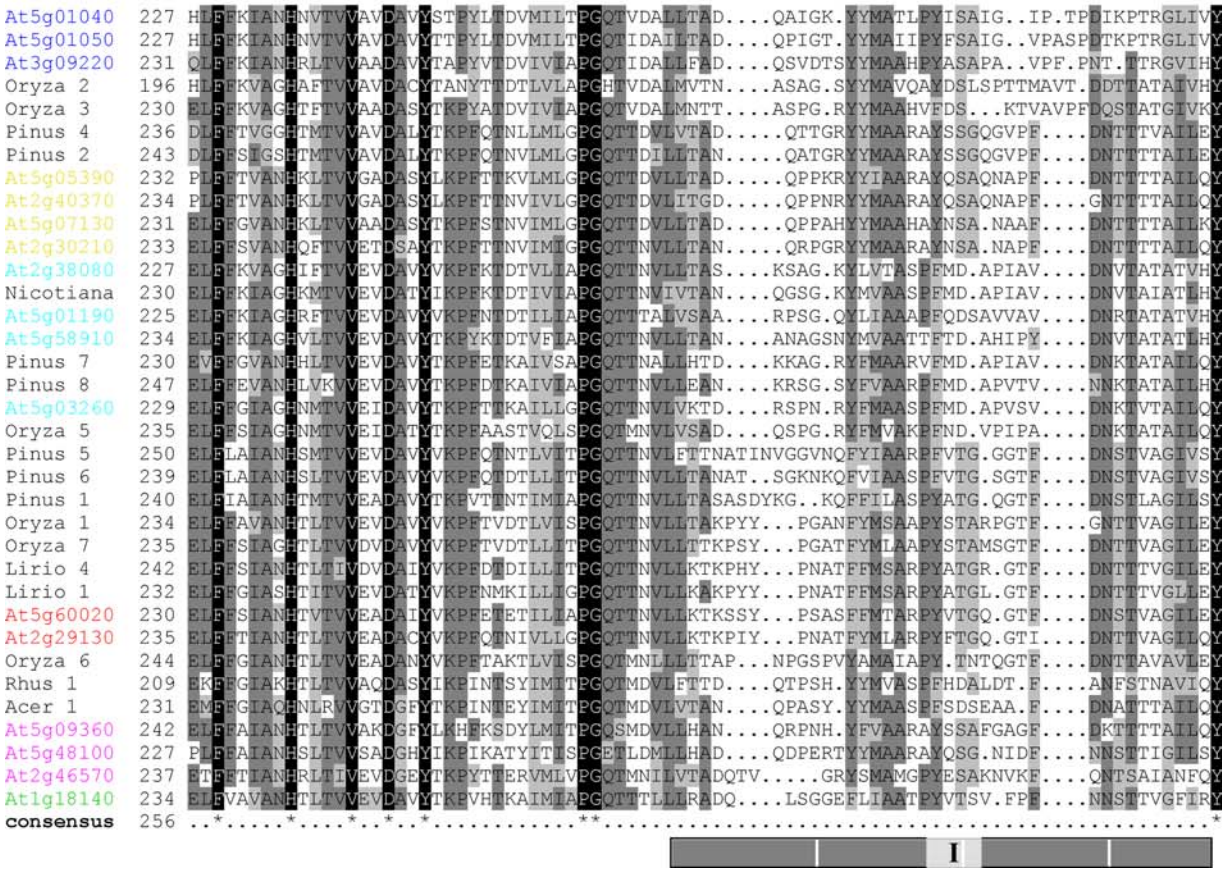


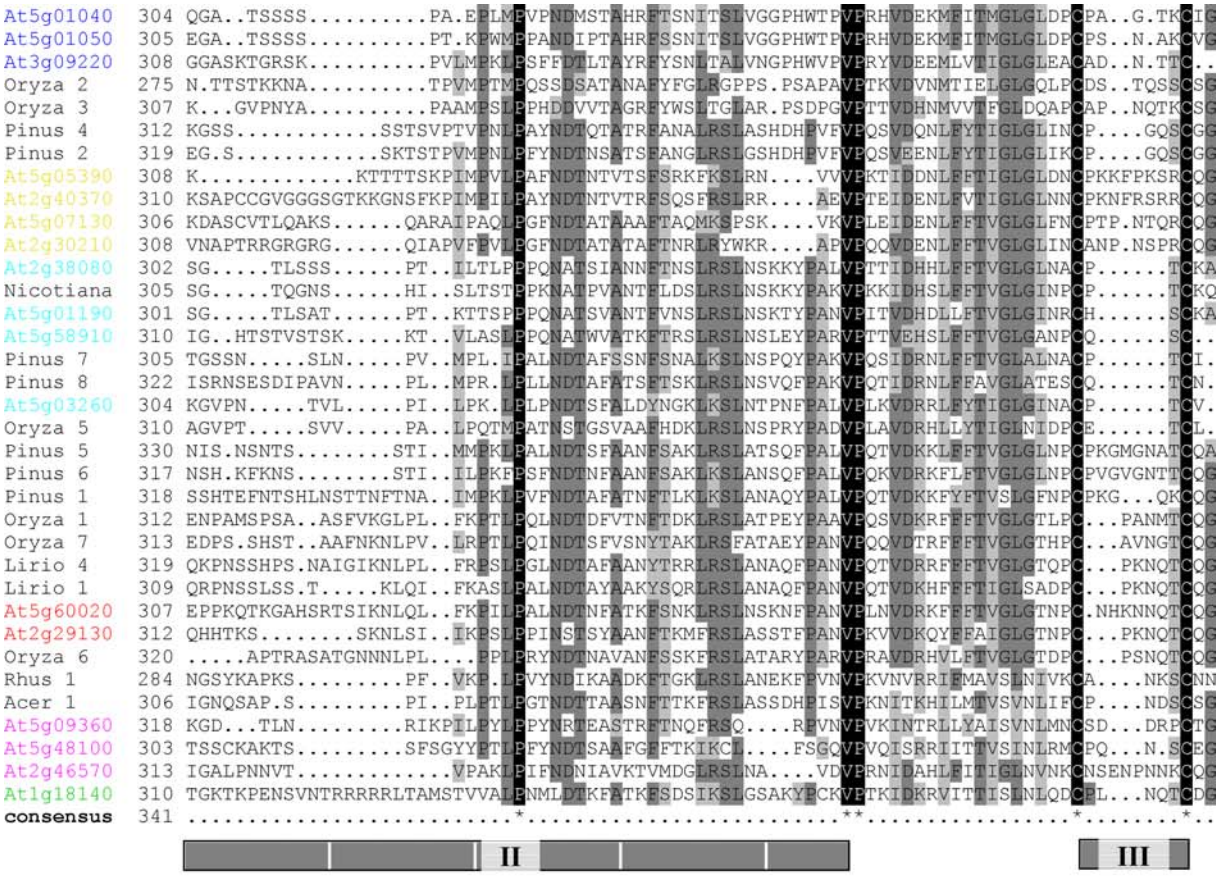
Fig. 1 Intron/exon structure of *Arabidopsis* LMCO gene family. Depicted intron positions were based on GenBank genomic sequence annotations. Exon color indicates phylogenetic grouping (*red* group 1, *light blue* group 2, *yellow* group 3, *lavender* group 4, *dark blue* group 5, *green* group 6)

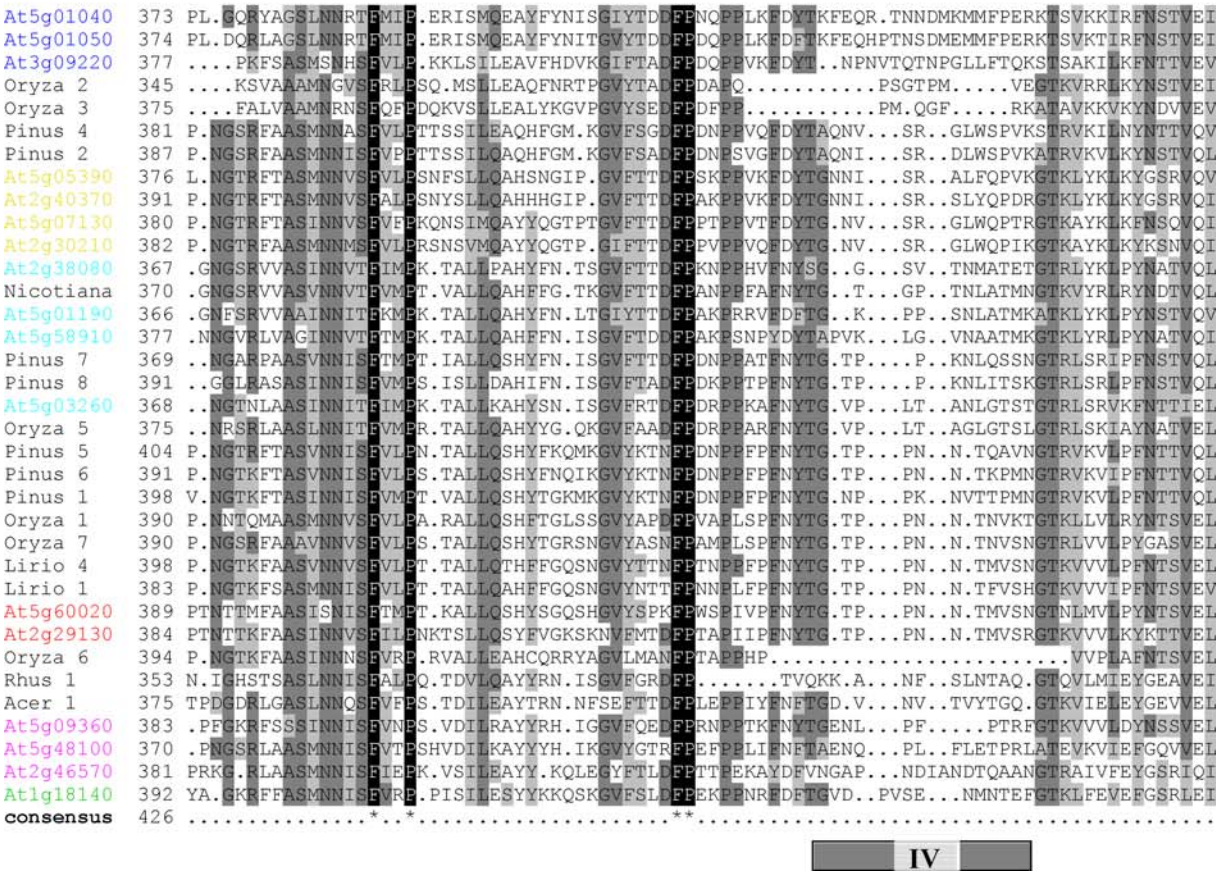
An amino acid alignment for a subset of the plant LMCOs, including all of the *Arabidopsis* genes, is shown in Fig. 2 with the predicted signal sequences identified by lower case letters. Putative signal sequences in pre-

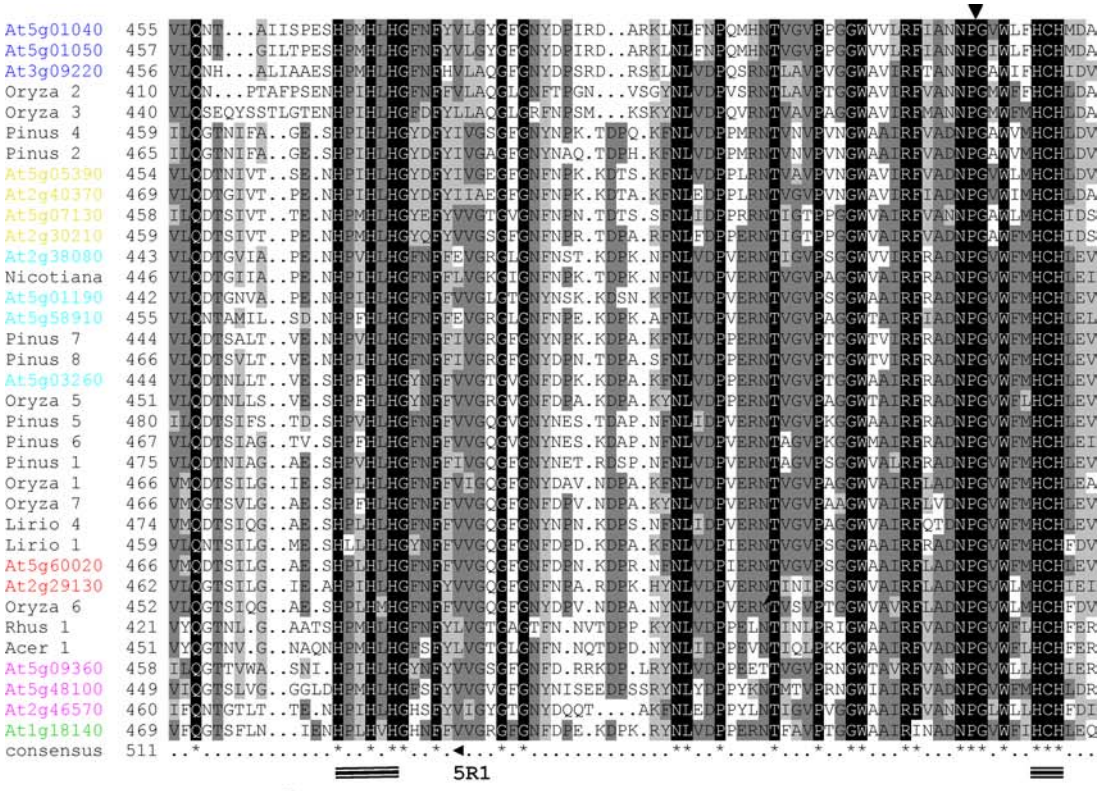

2F1
3F1

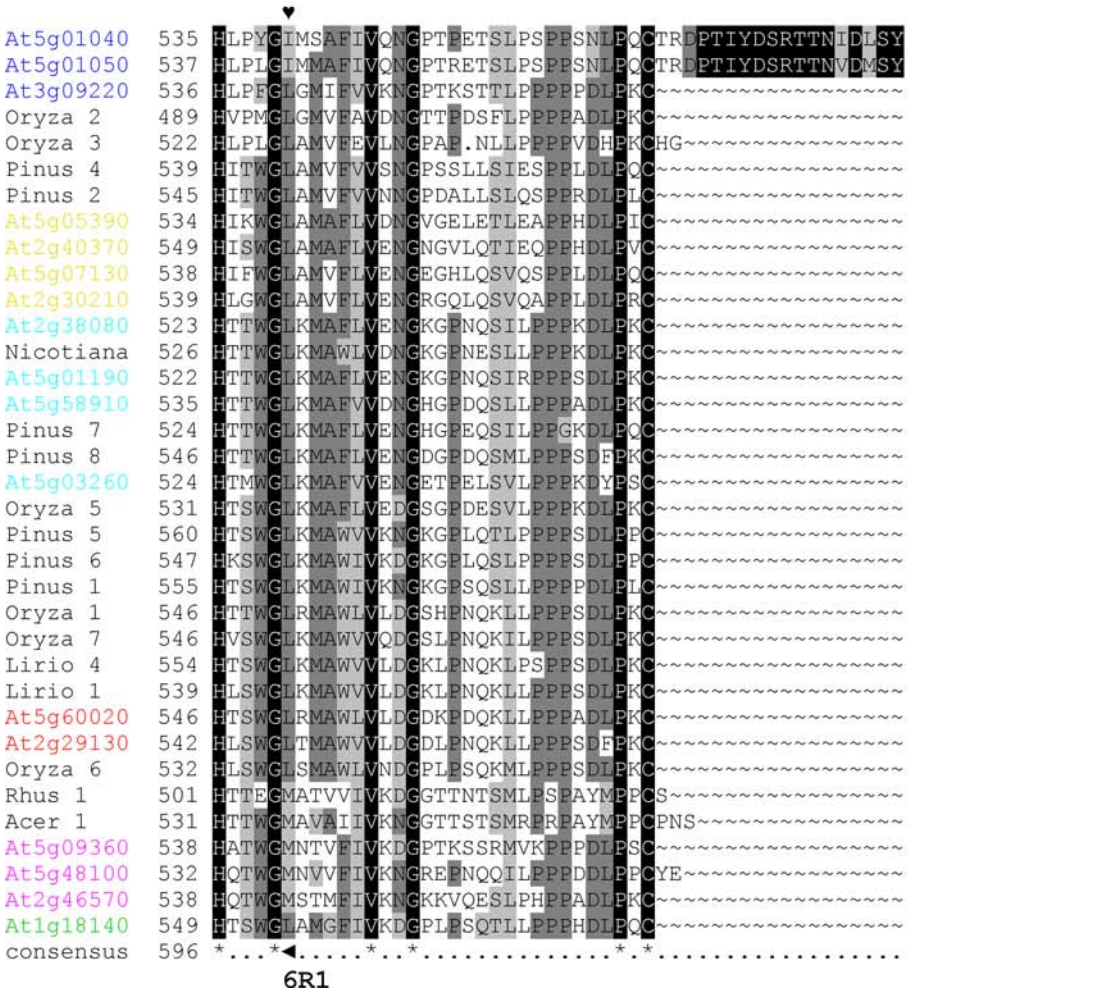

4F1
4F2


I


II
III


IV


5R1


6R1

◀

Fig. 2 Amino acid sequence alignment for plant LMCOs selected from GenBank. Similarities in predicted protein sequences for the LMCOs listed in Table 1 are *highlighted* in *gray-scale box* shading with *black* indicating residues that are identical in all 34 sequences. Predicted signal sequences are indicated by *lowercase letters*. *Numbered and shaded boxes* below the consensus sequence *highlight* the four regions of greatest internal sequence divergence. Phylogenetic groupings are denoted in color according to the same scheme as in Fig. 1. Sites corresponding to particular forward (▶) and reverse (◀) degenerate PCR primers are depicted in the consensus sequence and labeled immediately below. Other items of note include splice junctions (▼), copper-binding domains (≡), and an amino acid near the Type-1 copper site that may play an important role in modulating enzyme activity (♥). The figure was generated using MacBoxshade (version 2.15) written by Michael Baron.

dicted plant LMCOs were identified using *SignalP*, and cellular localization was predicted using *TargetP*. All of the genes for which predicted full-length sequences were obtained, with the exception of *Oryza sativa 2* (BAB92844.1), had putative N-terminal signal sequences ranging in length from 17 bp to 38 bp. The *Oryza* 2 accession may represent a partial sequence since it appears to be missing the first intron found in all other plant LMCOs. Most of the other predicted proteins contained signal sequences, suggesting they were secreted via the typical secretory pathway; however, *Pinus* 5 and *Oryza* 6 encoded LMCOs predicted to be targeted to the mitochondria.

All of the predicted enzymes share the strictly conserved N- and C-terminal copper-binding domains characteristic of LMCOs (Dean et al. 1998). This alignment also highlights a number of strictly conserved amino acids that have not been functionally characterized in previous studies of these enzymes. The second exon contains a strictly conserved proline-glycine-proline (PGP) motif, and there are strictly conserved tryptophan and aspartate residues, as well as a TQCP motif, downstream of the first copper-binding region. Immediately downstream of the second copper-binding domain are invariant arginine and glycine residues, and a stretch of approximately 45 highly conserved amino acids precedes the first region of high sequence variability. The C-terminal end of the LMCOs also exhibits sequence conservation between the third and fourth copper-binding domains due to a number of invariant residues, such as the NPGxW motif, and all of the LMCOs contain proline-rich C-termini.

The relative spatial relationships between conserved and variable domains are depicted in Fig. 3. The conserved areas around the copper-binding domains are readily apparent at either end of the protein by virtue of the high similarity scores for amino acid residues in these regions. The signal peptide and the central part of the protein are highly variable (low similarity scores) across the plant LMCO superfamily, both within and between phylogenetic groups. This divergent domain consists of four stretches of highly variable amino acid residues interspersed with conserved motifs (Fig. 2).

Phylogenetic analysis

The plant LMCOs examined in this study could be divided into six groups, five of which were strongly supported by bootstrap analysis (Fig. 4). It should be noted that group 4, comprised of *A. thaliana* At3g09220, At5g01040, At5g01050, and *Oryza sativa* 2, 3 and 4, is a loosely knit, basal cluster of genes, while At1g18140 has such a distinctive sequence that it segregated in its own group. Many of the species examined in this study had LMCO copies dispersed among multiple phylogenetic groups. *Arabidopsis thaliana* genes were identified in each of the five well-resolved phylogenetic groups, and *Populus trichocarpa* genes were identified in groups 1, 2 and 3. The gymnosperm sequences formed monophyletic clusters within groups, and the sequences from *Brassicaceae* species (*A. thaliana*, *A. procurrens* and *B. napus*) clustered together in groups 2 and 3. The species and gene phylogenies are not perfectly congruent within phylogenetic groups, however, as multiple clusters of *A. thaliana* genes were interspersed within each group. Considering that *B. napus* is a polyploid (Parkin et al. 1995), some of the closely related LMCOs in this species could conceivably have arisen as a result of polyploidization.

Since groups 1, 2 and 3 are composed of both gymnosperm and angiosperm LMCOs, it can be inferred that these groups are at least as ancient as the gymnosperm/angiosperm division (≈330 MYA; Martin et al. 1993). We were unable to use the degenerate primers to amplify LMCOs from plant species whose divergence predated the separation of seed plants from ferns. Since relatively few LMCOs recovered for this study fell into groups 4 and 5, the age of these gene classes remain ambiguous.

Predicted isoelectric points

It had been suggested previously that LMCO isozymes involved primarily with lignification of vascular tissues might be distinguished from isozymes serving other functions on the basis of their isoelectric points (pI) (Dean et al. 1998). The theoretical pI values calculated for the LMCOs analyzed in this study were highly consistent across all eleven group 1 enzymes (Fig. 5). With a mean value of 9.2 (0.61 SD), all of the enzymes in group 1 would be categorized as having a basic pI. Groups 2 and 3 also had relatively basic isoelectric focusing points. Groups 4 and 5, including the basal At2g46570 LMCO, demonstrated a great deal of heterogeneity in pI values. Out of the six LMCO groups, only groups 1 and 4 and groups 1 and 5 were deemed to have significantly different average pI values, based on a Bonferroni/Dunn test (0.05% level of significance).

Arabidopsis laccase expression

Preliminary northern analysis using 20 μg of total RNA showed that many of the *Arabidopsis* LMCOs

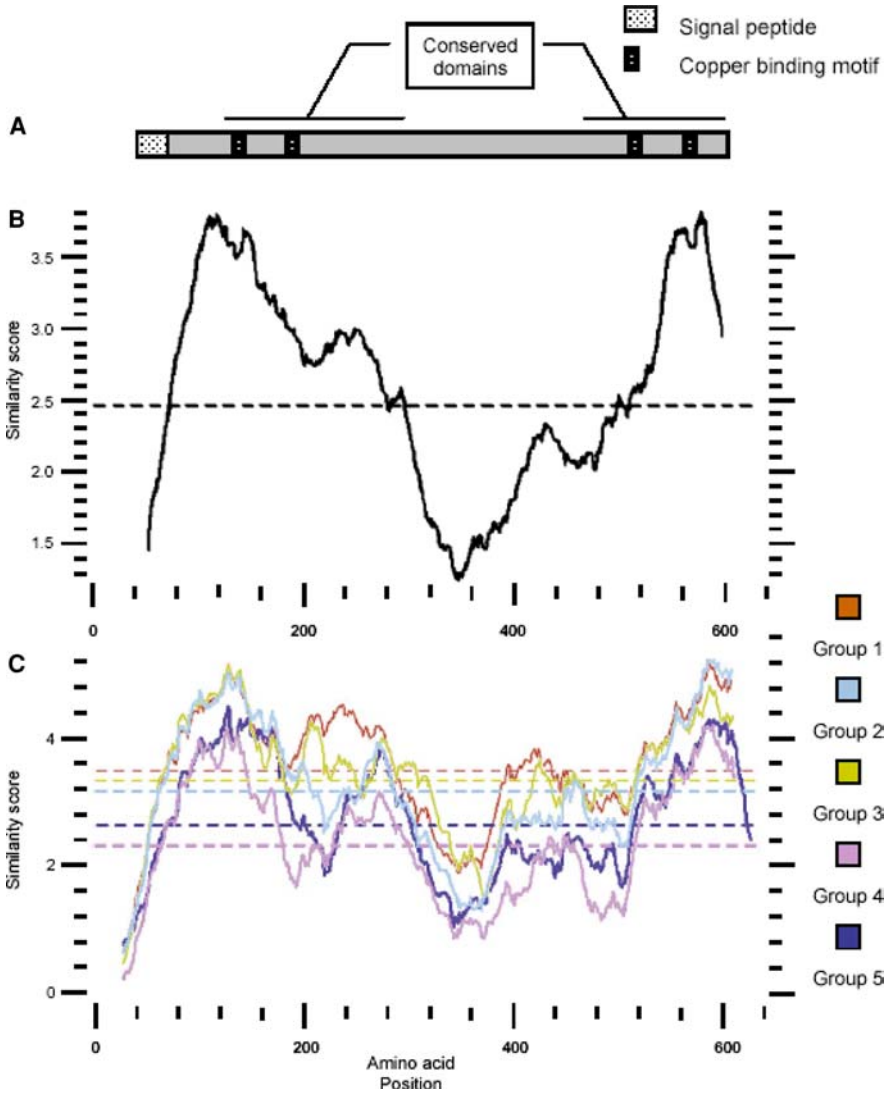


Fig. 3 Quantitative identification of conserved domains in LMCO proteins. Relative locations of the signal peptide and copper-binding domains in a typical LMCO are shown (**a**). Sequence similarity of LMCOs averaged across all phylogenetic groups shown in Table 1 was calculated along the length of protein using PLOTSIMILARITY (GCG) with window size = 100. The *dashed line* indicates the overall similarity score average (**b**). Average sequence similarity for each phylogenetic group was calculated with window size = 50. *Dashed lines* indicate the overall similarity score averages for each phylogenetic group. Data from group 6 was excluded since it is comprised of a single sequence (**c**)

were only expressed at extremely low levels (data not shown). To gain sufficient sensitivity with which to detect LMCO gene expression in a wide variety of tissues, while at the same time ensuring gene-specificity, RT-PCR with primers based on the 3′-untranslated regions of each LMCO transcript was used to assess LMCO expression in 13 *Arabidopsis* tissues. All of the *A. thaliana* LMCOs, with the exception of At5g58910, were expressed to some extent during normal development (Fig. 6). Although no RT-PCR amplification products were obtained for At5g58910 in this study, subsequent work has detected low levels of expression of this gene in roots (C-T. Wang, unpublished). Of the remaining LMCOs, some were expressed at relatively high levels in all tissues, regardless of developmental stage, whereas others were regulated in a developmental or tissue-specific manner. Genes found to be expressed at consistently high levels in all tissues included At2g29130 (group 1), At5g03260 (group 2), and At2g40370 (group 3). Others, such as At5g60020 (group 1), At2g38080 (group 2), At5g05390 (group 3), and At2g46570 (group 4), were predominantly expressed in the inflorescence stem, and At5g05390 in particular was limited almost exclusively to the stem. Other genes (At5g48100, group 4; At2g30210, group 3; At3g09220, At5g01040 and At5g01050, group 5) were more highly expressed in tissues other than the inflorescence stem. At2g30210 was only expressed in roots and appeared to be most abundant in immature roots.

Individual *Arabidopsis* LMCO genes were also differentially regulated during organ development. At5g01040 was expressed along a developmental gradient in the inflorescence stem, progressing from moderately high levels in the basal, older internode 1 to weak levels in internodes 2 and 3. No transcript accumulation was evident in the youngest parts of the stem (internode 4 and terminal internode). Conversely, At5g48100 expression in the inflorescence stem was lowest in the basal stem tissues and highest in the young, developing tip. Similarly, in the basal rosette, At2g38080 transcript levels diminished during leaf development, whereas

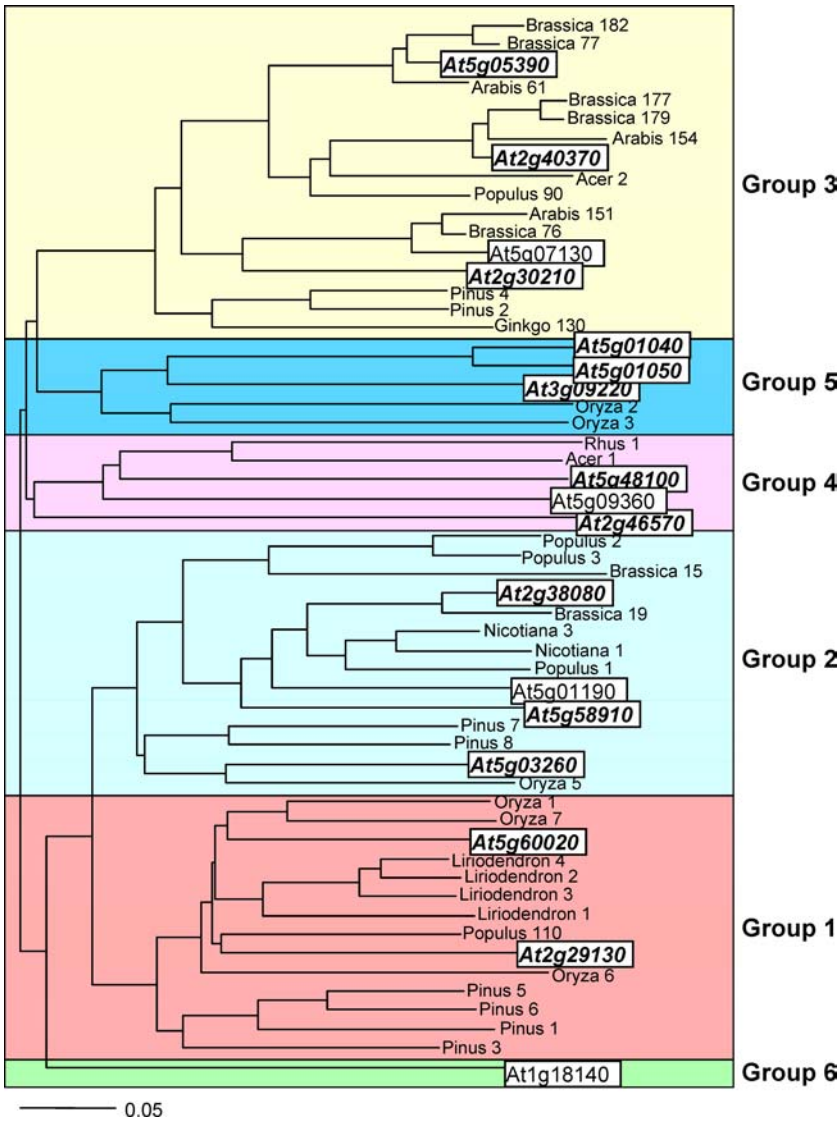


Fig. 4 Neighbor-joining phylogeny depicting relatedness of plant LMCOs. Predicted transit peptides were removed prior to analysis. Arabidopsis genes are shown in *boxes*; *boldface italics* indicate those genes whose products were examined in the expression analyses

At5g48100 transcript levels increased during the same developmental progression.

Genome-scale transcriptional profiling data for *Arabidopsis* generated using massively parallel signature sequencing (MPSS) has recently been made available online (http://mpss.udel.edu/at/, Meyers et al. 2004), and Table 5 shows the MPSS data for all 17 *Arabidopsis* LMCO genes from a selection of normal *Arabidopsis* tissues. MPSS detected at least some level of expression for all 17 LMCO genes. Notably, MPSS found only very low levels of expression for At5g07130 and At5g09360, two of the genes whose expression we could not detect by PCR. The other gene whose expression was not detected by PCR, At5g01190, also had low expression according to the MPSS data, although it was within a range comparable to other genes for which PCR signals were detected. Both techniques were in agreement that expression of At2g46570 was low in all situations tested. Two genes, At2g40370 and At5g48100, showed low expression in the MPSS data, but moderate to high expression according to PCR. In the case of At5g48100, the highest expression levels were found in young seedlings and open flowers, neither of which was a specific tissue type analyzed by MPSS. PCR analysis suggested At2g40370 transcripts to be abundant and nearly constitutive in their distribution, so it is not clear why MPSS detected fairly low levels of this transcript limited to root tissues. However, attempts to draw fine-scale comparisons between results from these two techniques should be considered with caution due to the sensitivity and non-linearity of PCR amplification technique used in this study, as well as the fact that the tissue samples used in the MPSS study and the work reported here were not the same.

Discussion

The initial premise for this study was the idea that specific members of the LMCO gene family might catalyze

Phylogenetic Group	Gene	Calculated pI
1	Liriodendron 1	9.3
	Liriodendron 2	9.5
	Liriodendron 3	9.3
	Liriodendron 4	9.6
	At5g60020	9.3
	Oryza1	7.4
	Oryza 6	8.9
	Oryza 7	7.0
	Pinus 1	9.5
	Pinus 3	9.5
	Pinus 5	9.4
	Pinus 6	9.5
	Populus 110	9.2
	At2g29130	9.6
2	At5g01190	9.5
	At5g58910	9.1
	At5g03260	8.9
	Oryza 5	7.1
	Pinus 8	7.7
	Populus 3	8.3
	At2g38080	9.4
	Pinus 7	8.9
	Nicotiana 3	9.5
3	At5g05390	9.3
	Populus 90	9.4
	Pinus 2	8.5
	Pinus 4	7.3
	At2g40370	9.1
	At2g30210	9.6
	At5g07130	7.0
4	Acer	5.4
	At5g48100	7.1
	Rhus	6.8
	At5g09360	9.6
5	At3g09220	9.1
	At5g01040	8.1
	At5g01050	7.1
	Oryza 3	6.1
	Oryza 4	5.6
6	At1g18140	9.1

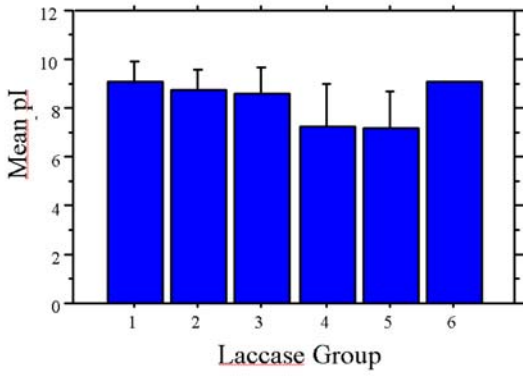


Fig. 5 Isoelectric focusing points (pI) for selected plant LMCOs. With the exception of the truncated protein predicted from the partial *Rhus vernicifera* cDNA, the pI values were calculated for full-length, mature protein sequences using the ProtParam tool (http://us.expasy.org/tools/protparam.html). Mean and standard deviations of calculated pI of each of the LMCO phylogenetic groups were found to be statistically significant at the 0.005% level for groups 1 and 4 and groups 1 and 5

the polymerization of specific lignin precursors in various cell types at different stages of plant development. The complete and annotated *Arabidopsis* genome sequence provided a framework gene family against which other LMCO genes could be compared. Results from this work showed that LMCOs constitute a large and diverse family in plants, in which gene family members exhibited a broad variety of expression patterns. Phylogenetic analysis suggests that LMCOs are widespread, if not ubiquitous across higher plant families, with multiple gene copies occurring in each species. Though the ancient duplications that initially established at least three of the identified LMCO groups have been maintained through time, LMCOs have also experienced more recent gene duplication events. In *Arabidopsis*, individual LMCOs were found to be either developmentally or constitutively expressed, and they were identified in a variety of plant organs, including some that are not considered to contain significant amounts of lignin.

Prior to this, plant LMCOs have been primarily classified according to isoelectric focusing point (pI), with the implicit assumption that pI may bear some relationship to substrate kinetics and enzyme function (O'Malley et al. 1993; Liu et al. 1994; Dean et al. 1998). When averaged across each monophyletic gene cluster, significant differences in average pI values were only seen in comparisons of groups 1 and 4 and groups 1 and 5. Given evidence that the estimated pIs vary little between most of the known plant LMCOs, this particular characteristic appears unlikely to be of use in identifying LMCO function or providing a reliable means of enzyme classification. Since expression profiles were found to vary between phylogenetic groups, it seems best to categorize plant LMCOs on the basis of sequence similarity and phylogenetic clustering until such time as specific physiological functions are defined.

Most of the diversity between LMCOs lies in the first exon, which is principally composed of the signal sequence, and in the central portion of the protein, between the second and third copper-binding domains. Despite the high degree of variability in the signal peptide sequence, most laccases are predicted to be secretory proteins. The middle region of the protein contains four highly variable domains ranging from 3 to 59 amino acids in length and separated by islands of conserved sequence (Fig. 2). The length of the variable regions differs between LMCOs both within a single species and within phylogenetic groups. The proline-rich nature of the central variable regions and the differences in sequence length may indicate that these sequences contribute to surface loops in the tertiary structure of the folded protein.

As expected, there was considerable sequence conservation within the four copper-binding domains (Figs. 2, 3). One of the few exceptions was the *Oryza* 6 gene where a leucine to methionine substitution exists within the third copper-binding domain. It may be of interest that this was also one of the two genes encoding

►

Fig. 6 Expression levels of *Arabidopsis* LMCOs in various tissues and developmental stages. Messenger RNA isolated from the identified tissues was reverse transcribed, and 20 ng of cDNA was used as template input for each PCR reaction. Genomic DNA controls showing amplification across introns demonstrate the absence of genomic DNA contamination in the cDNA samples. The genes are clustered according to phylogenetic group. Actin was included as a control for equal cDNA input between tissues

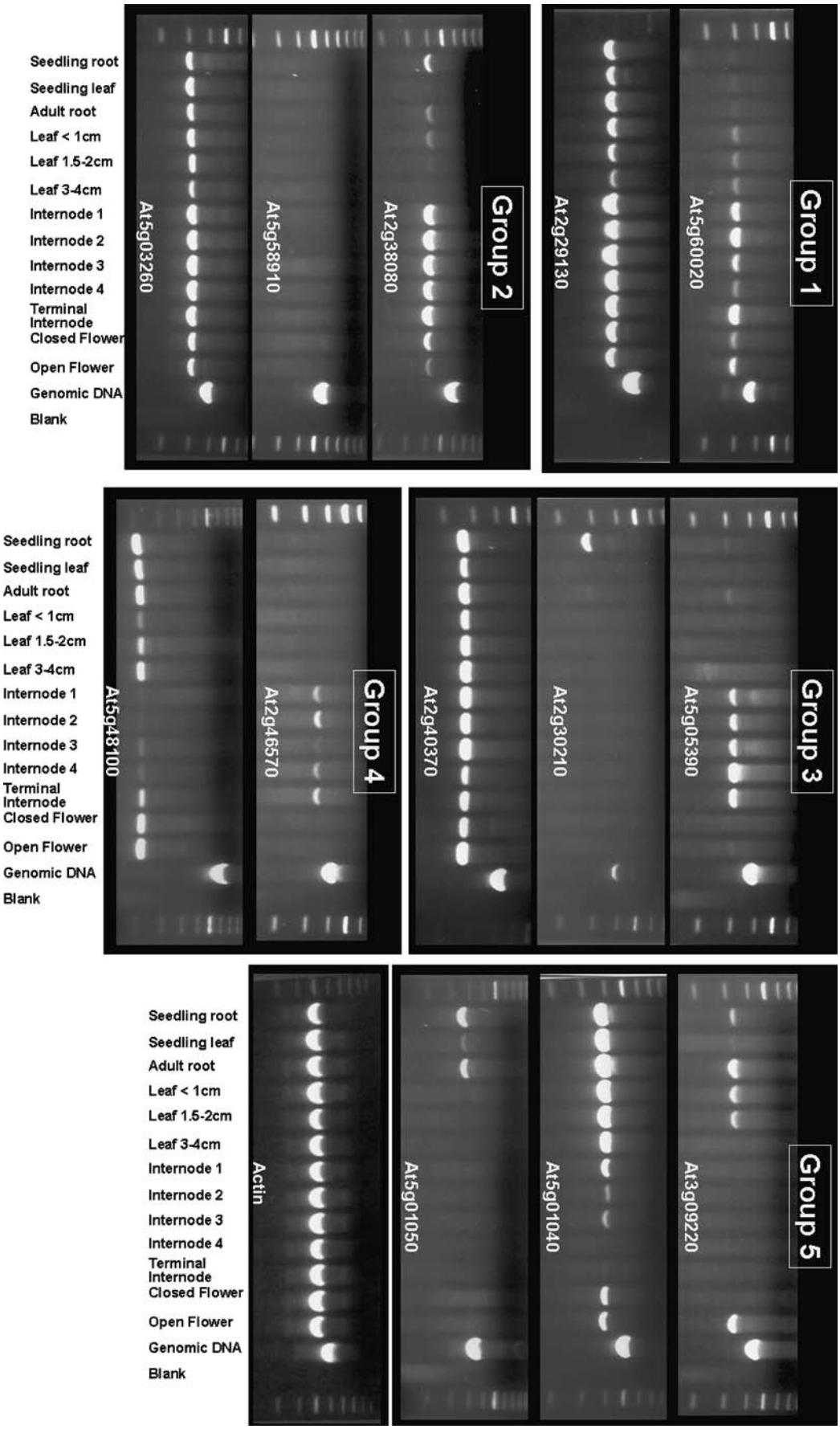

Group 1
At2g29130
At5g60020
Group 2
At5g03260
At5g58910
At2g38080
Seedling root
Seedling leaf
Adult root
Leaf < 1cm
Leaf 1.5-2cm
Leaf 3-4cm
Internode 1
Internode 2
Internode 3
Internode 4
Terminal Internode
Closed Flower
Open Flower
Genomic DNA
Blank
Group 3
At2g40370
At2g30210
At5g05390
Group 4
At5g48100
At2g46570
Seedling root
Seedling leaf
Adult root
Leaf < 1cm
Leaf 1.5-2cm
Leaf 3-4cm
Internode 1
Internode 2
Internode 3
Internode 4
Terminal Internode
Closed Flower
Open Flower
Genomic DNA
Blank
Group 5
At5g01050
At5g01040
At3g09220
Actin
Seedling root
Seedling leaf
Adult root
Leaf < 1cm
Leaf 1.5-2cm
Leaf 3-4cm
Internode 1
Internode 2
Internode 3
Internode 4
Terminal Internode
Closed Flower
Open Flower
Genomic DNA
Blank

Table 5 LMCO gene expression in selected *Arabidopsis* tissues from massively parallel signature sequencing (*MPSS*)

Gene Model	Tissue					
	Callus	Inflorescence	Leaf (21 day)	Root (21 day)	Silique (24–48 hpf *)	Root (21 day)
1g18140	0	44	0	22	0	12
2g29130	0	0	2	90	3	14
2g30210	0	0	4	191	0	70
2g38080	0	297	8	73	86	84
2g40370	0	0	0	0	0	11
2g46570	0	5	0	3	9	0
3g09220	0	3	7	107	0	94
5g01040	269	0	3	63	0	90
5g01050	269	0	3	63	0	90
5g01190	0	8	1	25	11	0
5g03260	0	12	4	14	4	25
5g05390	0	0	3	10	24	0
5g07130	0	0	0	0	0	2
5g09360	0	0	0	0	15	0
5g48100	0	0	0	6	0	0
5g58910	0	3	7	107	0	94
5g60020	0	122	3	45	36	0

These data, collected from the Arabidopsis MPSS website (http://mpss.udel.edu/at/), represent the number of times the predominant signature tag for each *Arabidopsis* LMCO gene was seen in a particular MPSS library. As per details available from the website, MPSS libraries were typically characterized for 2.0–3.0×10^6 tags
**hpf* hours post-fertilization

LMCOs predicted to possibly localize to mitochondria. Perhaps of greater note was the Met residue at position 601 in the consensus sequence distinguishing the group 4 LMCOs from all other members of the gene family. By virtue of its immediate proximity to the Type-1 copper site, which is where electrons from reduced substrates are passed to the enzyme, this residue has been suggested to be involved in determining redox potential and substrate specificity of plant and fungal LMCOs (Palmer et al. 1999; Xu et al. 1999). A number of conserved motifs not directly involved in copper binding were identified in this study, and detailed studies of these sites using site-directed mutagenesis are expected to provide new insights into the functioning of these enzymes.

Contrary to previous suggestions that LMCO genes may be limited to a handful of higher plant families (Mayer and Staples 2002), phylogenetic analysis suggests that they are widespread amongst angiosperms, and quite probably in gymnosperms as well. The monophyletic clustering of LMCO genes from such wide-ranging taxonomic groups as gymnosperms (Ginkgoaceae, Pinaceae), monocots (Poaceae) and numerous divergent angiosperm families (Anacardiaceae, Brassicaceae, Magnoliaceae, Solanaceae, Salicaceae, Sapindaceae) argues strongly against multiple, independent origins of the LMCO superfamily within plants. A much more likely scenario is simply that LMCOs are widespread in the plant kingdom and that additional genes will come to light with additional study. Although we were unable to gather evidence for LMCO genes in non-vascular plants, the existence of multiple gene classes in fungi, yeast and bacteria suggests that LMCOs are ubiquitous to all kingdoms and that they will be found in primitive plants as well.

From the available data, the six phylogenetic groups of LMCO genes appear to be of relatively ancient origin, dating back at least to the angiosperm/gymnosperm divergence. The pattern of differentiation suggests that relatively ancient duplications, such as the multiple Pine genes in groups 1, 2 and 3, have been retained over time. Furthermore, new gene duplications have repeatedly arisen, such as the multiple clusters of group 3 *Brassica*, *Arabis* and *Arabidopsis* LMCOs and the tandemly duplicated, highly homologous (89% identical) At5g01040 and At5g01050 *Arabidopsis* genes in group 5. The maintenance of both ancient and recent gene duplications suggests that there is both purifying selection acting to conserve gene functions common to a wide range of plant species and diversifying selection resulting in new gene function over time (Li 1997; Clegg et al. 1997). New gene functions may relate to differences in subcellular localization, as with the possible localization of the *Oryza* 6 and *Pinus* 5 LMCOs to mitochondria, as well as differences in tissue-specific expression or substrate preferences.

Previous studies of plant LMCOs have focused on enzymes expressed predominantly or exclusively in differentiating xylem, particularly in tree species (*P. taeda*, *A. pseudoplatanus*, *P. trichocarpa*, and *L. tulipifera*) distinguished by dedicated production of secondary xylem. In these studies, LMCO gene expression was detected by northern blot analysis or inferred from chromogenic reactivity in sectioned stems or from purified enzyme. Thus, northern blot analysis of *P. trichocarpa* LMCO genes demonstrated exclusive expression in stem tissues (Ranocha et al. 1999). Chromogenic detection of LMCO activity in *P. taeda* demonstrated expression in differentiating xylem and callus tissue, but not embryos, megagametophytes or pollen strobili (Bao et al. 1993). However, northern blot analyses of eight *P. taeda* LMCO genes demonstrated that at least one of these genes (LAC7) was expressed in a wide variety of tissues, including roots, needles and shoot tips (Sato et al. 2001). Differences in starting materials and methods of LMCO detection make it impossible to draw strong conclusions from these studies. Although the

small volume of cells that make up the vascular tissues of herbaceous plants, such as *Arabidopsis*, could make detection of LMCO expression difficult if the enzyme were limited to these tissues, RT-PCR analysis provided sufficient sensitivity to make a true test whether or not LMCO expression was vascular tissue-specific.

To assess gene expression across a broad range of organs at different developmental stages in *Arabidopsis*, gene-specific RT-PCR was performed on seedling roots and leaves and on adult roots, rosette leaves of different sizes, individual inflorescence stalk internodes, which contain vascular cells in a range of developmental states from mature vessel elements at the base to immature elements at the tip of the stem, and both closed and open flowers. Although not strictly quantitative, this RT-PCR technique provides sufficient relative information to demonstrate differential regulation of LMCO genes in these samples, and the results were more or less in agreement with the more quantitative data obtained using MPSS. In both cases, the data suggest that LMCO function in *Arabidopsis* likely extends well beyond lignification. The expression profiles appeared to fall into three general patterns. First, there was At5g58910 for which no expression was detected in this study, although expression of this gene has been detected in more recent studies of seedling roots (C-T. Wang, unpublished). A second class of LMCOs, exemplified by At2g40370 and At5g03260, were found to be expressed constitutively in all organs at all stages of development, while a final group of LMCOs, such as At2g38080 and At2g30210, showed regulated expression in a tissue-specific manner. If these enzymes were exclusively involved in lignification, gene expression would be strongly correlated with vascular tissue differentiation, and constitutive expression would not be expected.

The most important finding from this study was that different LMCO genes are expressed in a wide array of patterns within a single plant species. Proposed explanations of functional significance for the multiple LMCO isoforms detected in previous studies have focused primarily on lignification. Thus, differential affinity for the coniferyl and syringyl alcohol precursors of lignin (Dean et al. 1998), differential expression during the development of various xylem and phloem elements (Dean et al. 1998), and functional diversification to address specific defense or stress responses (Mayer and Staples 2002) have all been proposed as functions for LMCO isozymes. Our observations of LMCO gene expression patterns in *Arabidopsis* did not find strong correlations between lignification and all LMCO family members, although at least one family member (At2g38080) showed inflorescence stalk expression in a pattern consistent with the degree of lignification of vascular tissues in this organ. Constitutive expression of the LMCO genes, as well as tissue-specific expression in minimally lignified tissues, such as young roots (e.g. At2g30210), in fact, suggest that a majority of the LMCO gene products in *Arabidopsis* are likely involved in physiological functions other than lignin deposition.

Wound healing through oxidative polymerization of alkylcatechols in the sap of *Rhus* species (true laccase; group 4) had been the only strongly supported physiological function advanced for plant LMCOs besides lignification. However, we recently presented evidence for ferroxidase activity in a plant LMCO and discussed the possibility that these enzymes might have the capacity to act in iron-uptake systems similar to the FET3 system in *Saccharomyces cerevisiae* (Hoopes and Dean 2004). In yeast, two distinct, but highly homologous, LMCOs (FET3 and FET5) function in iron transporters localized to the plasmalemma and tonoplast membrane, respectively (Urbanowski and Piper 1999). Tissue-specific expression and subcellular localization requirements for precise mobilization of iron between cellular compartments in plants could easily underlie the diversity noted in the LMCO gene family. Such a physiological function for these plant enzymes would provide a satisfying explanation for their apparent association with lignifying vascular tissues (Bao et al. 1993; Liu et al. 1994; LaFayette et al. 1999; Ranocha et al. 1999). Activation of LMCO-dependent iron pumps may be required to scavenge free iron from extracellular spaces so as to minimize the potential for free-radical production from the reaction of free iron with the H_2O_2 produced for the lignification process. Alternatively, the ability of microbial enzymes related to LMCOs to oxidize copper (Shi et al. 2003) or manganese (van Waasbergen et al. 1996; Brouwers et al. 1999) suggest that some plant LMCO gene family members could play roles in the metabolism of other essential metals. Further studies should focus on determining the cell-specific expression and subcellular localization patterns of the different LMCO gene family members during development and under a variety of stress regimes, in addition to examining an expanded selection of potential inorganic substrates.

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